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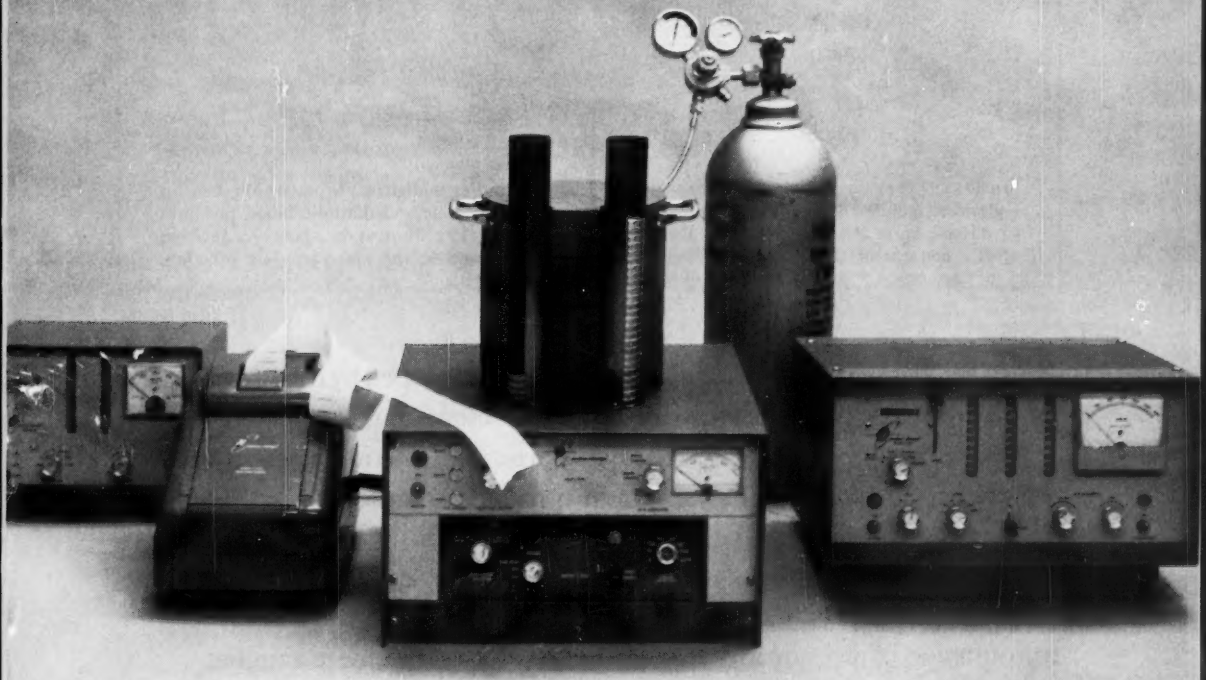

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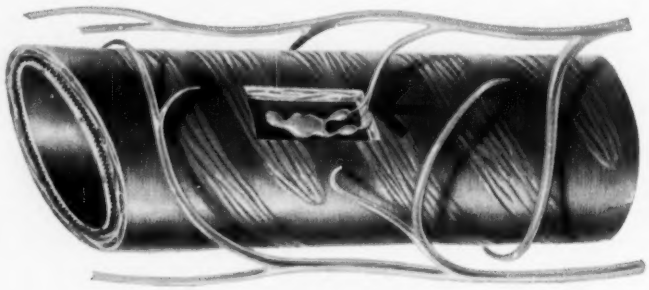

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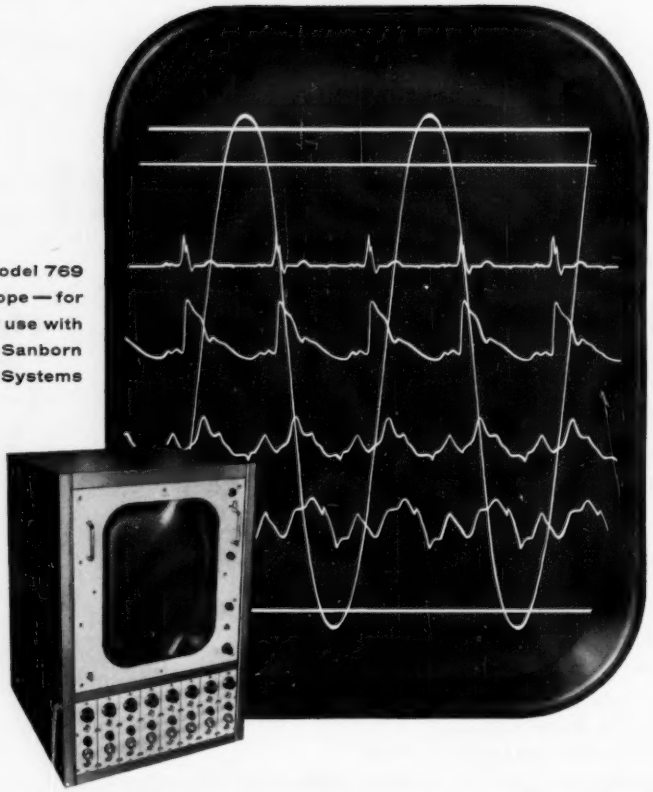

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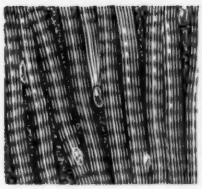

IN AUGUST—(1881)—at the fourth annual meeting of the American Society of Microscopists, Dr. Jacob Redding of Falmouth, Indiana takes issue with the belief that muscular contraction is due to changes in non-living formed material and is not dependent upon bioplasm. He cites considerable histological evidence to show that "every muscular contraction is directly dependent on active and forcible changes taking place in confined bioplasm."[1]

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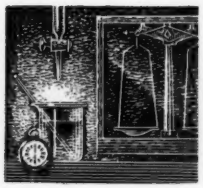

IN AUGUST—(1912)—there is reported an improved method for the determination of tyrosine in protein. Folin and Denis[2] show that the gravimetric separation of tyrosine from protein hydrolysis products is not quantitative. They have developed a procedure which involves a 12-hour digestion in acid followed by development of a blue color with a phosphotungstic-phosphomolybdic reagent. Results are consistently higher than those obtained with the gravimetric methods used in the past.

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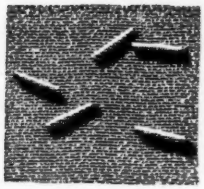

IN AUGUST—(1943)—the Archives of Biochemistry carries a paper by Lauffer and Stanley describing the effects of urea upon tobacco mosaic virus. Neutral 6M urea solutions disintegrate high molecular weight virus nucleoprotein particles into much smaller fragments. Phosphorus analyses indicate that nucleic acid is split away from protein. Much of the virus infectivity is lost. The considerable reduction in the specific infectivity of residual high molecular weight nucleoprotein indicates that inactivation may take place before the molecule is extensively disintegrated.[3]

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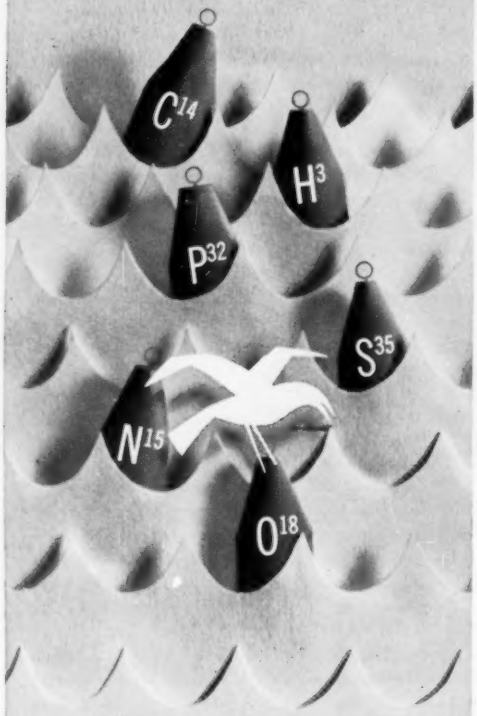

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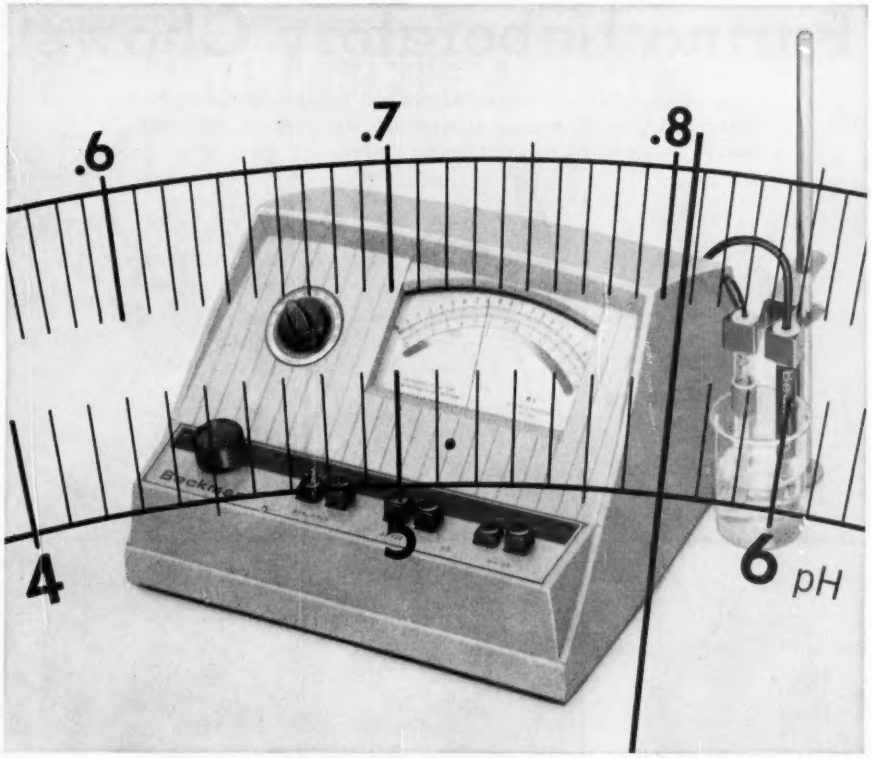

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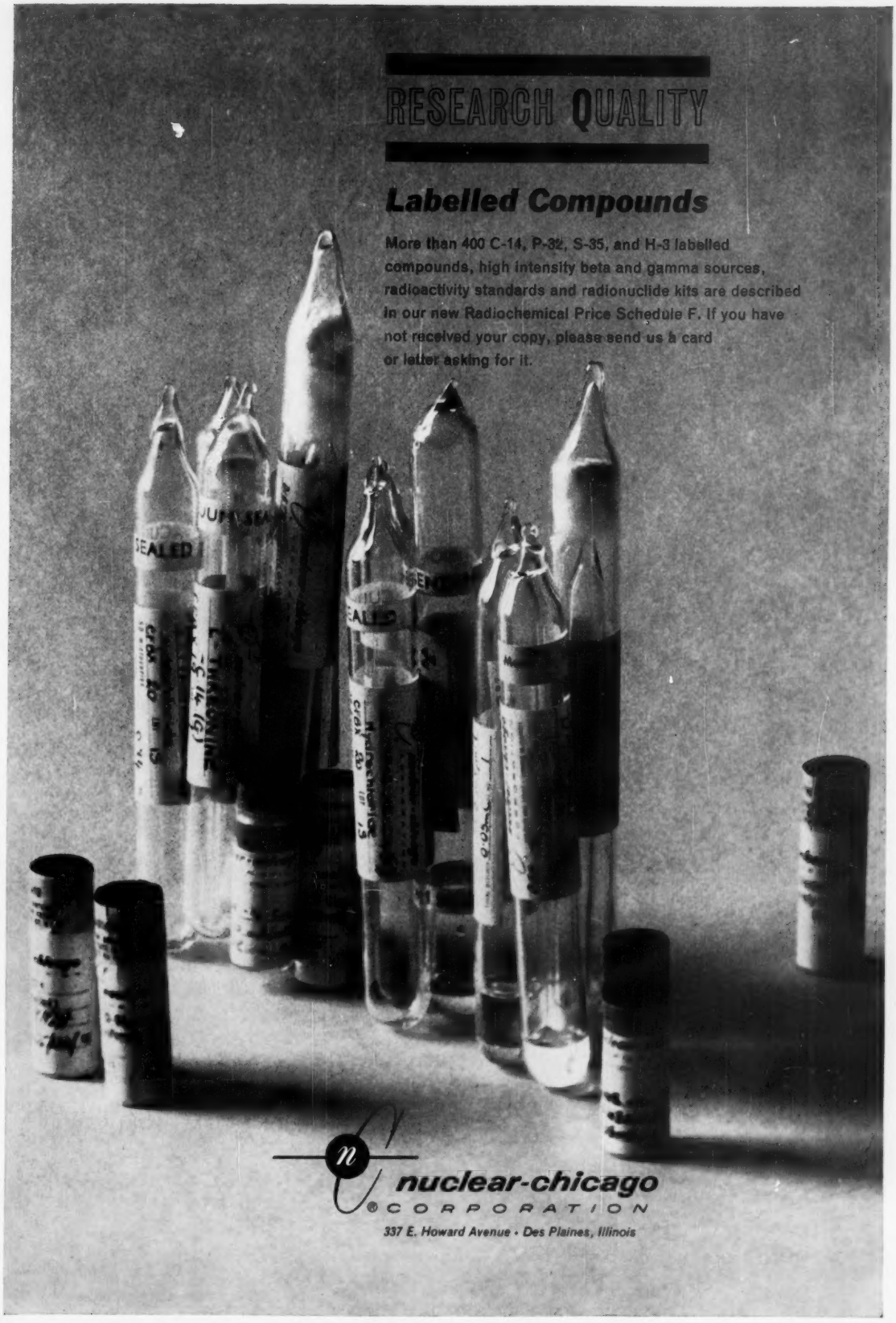
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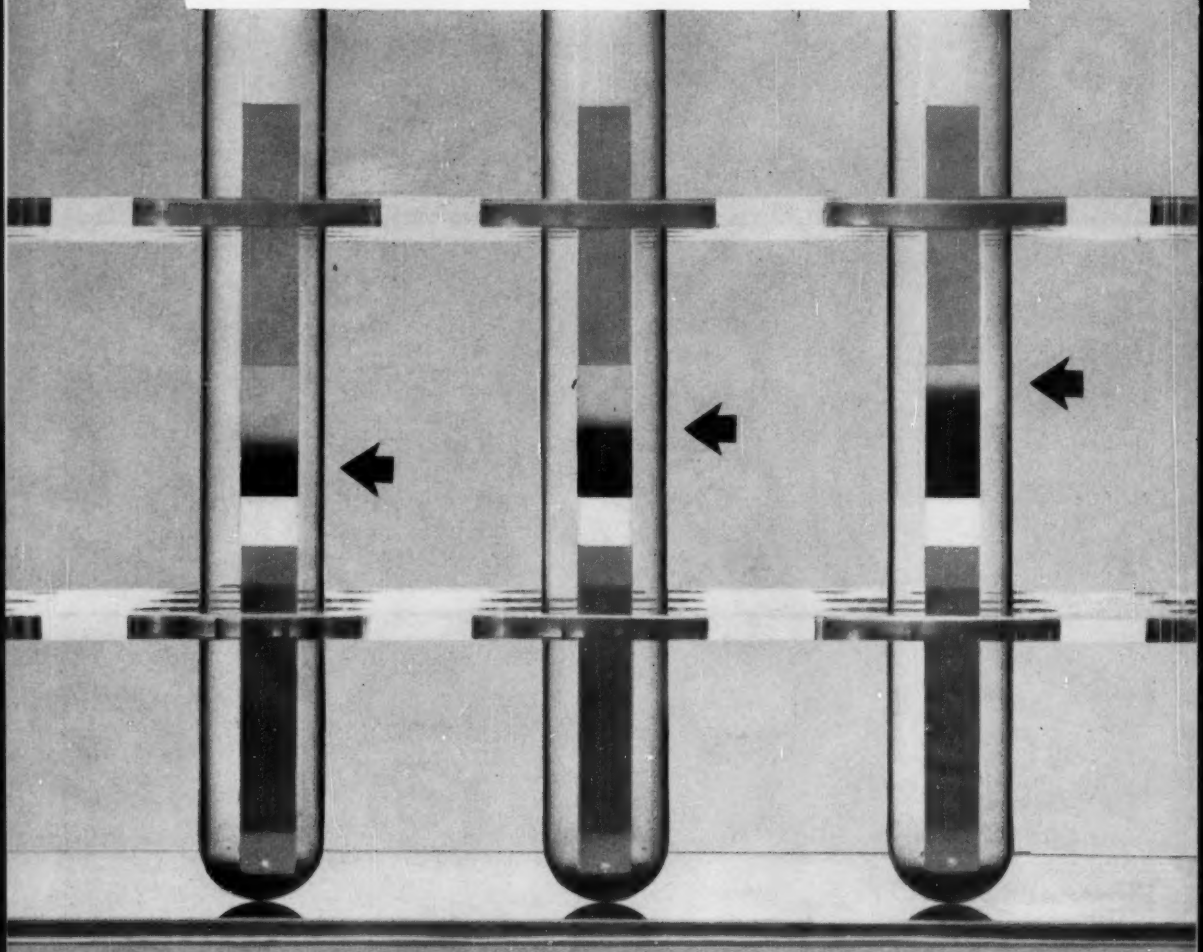

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MEASUREMENT OF BLOOD FLOW IN THE COLLATERAL VESSELS OF THE PORTAL VEIN; PRELIMINARY RESULTS OF A NEW METHOD

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The portal vein and its branches may be demonstrated by the intrasplenic injection of radiocontrast media. Such identification of collateral vessels aids understanding of some of the symptoms of chronic liver disease (1–4). The size and location of collateral vessels is shown; however, the blood flow through them is not measured. Certain syndromes, such as the neurological complications of chronic liver disease (portal systemic encephalopathy), depend more on the total collateral blood flow than on the presence of any specific collateral vessels (5). This paper reports a method for measuring the portal collateral blood flow and its application in 12 patients.

If an indicator substance is introduced into, and is rapidly absorbed from the spleen, part of it reaches the general circulation by passing through the liver and the remainder by passing through any collateral channels present (Figure 1). The amount passing by each of the two routes is directly proportional to its respective blood flow. The amount passing through the liver is measured from the concentration of the indicator in the hepatic venous blood and the blood flow through the liver. Some indicator recirculates to the portal vein by way of the arteries and is measured in the femoral artery. When absorption is complete the total amount of the indicator absorbed may be determined from the product of its concentration and its volume of distribution.

During the time required to reach equilibrium, the difference between the amount of radioactivity leaving the splanchnic bed and the amount entering should be equal to the effective radioactivity injected minus the residual radioactivity in the splanchnic plasma. The radioactivity leaving the splanchnic bed is that leaving by the hepatic vein plus that leaving by the collaterals.

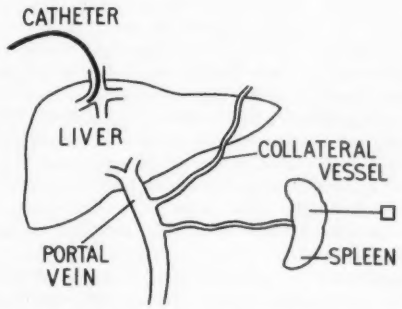

Fig. 1. Diagram of the experimental method. HISA is injected into the spleen and may reach the peripheral circulation by passing through the liver or through the collateral vessels. HISA passing through the liver may be measured by means of a hepatic vein catheter.

METHOD

Assumptions. The major assumptions of the method are listed below in the same order in which they appear in the method and the evaluation of the data. Continuous sampling from the hepatic vein must extend throughout the period of absorption of indicator from the spleen. The indicator must mix completely with the blood in the splenic vein before the first branching point and with all blood subsequently entering the splenic vein. The sampling from the hepatic vein must be uniform and must not alter the blood flow in the hepatic vein. The sulfobromophthalein (BSP) technique must measure blood flow in the hepatic vein. Recirculated indicator must take the same route as injected indicator and this route must not vary during the study. The time of circulation from heart to hepatic vein is the same as circulation from heart to femoral artery. The splanchnic blood volume may be estimated from values in the literature. The flow of significant indicator through collateral circulation

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is slow compared with the flow through the hepatic vein. The total blood flow through the liver must not change during the procedure.

A preliminary investigation was carried out to test the suitability of human iodinated serum albumin (HISA) as an indicator. The HISA was prepared by the technique of McFarlane (6) and had an activity of 5 to 25 μc per ml. A measured dose of HISA in 1 to 2 ml was injected into the spleen and samples were taken from a peripheral artery or vein for 20 minutes to determine concentration. The plasma volume was determined with Evans blue. Eight patients with splenomegaly were studied; 6 had portal hypertension. The time necessary to reach a constant concentration was from 2 to 3.5 minutes (mean, 2.8 minutes) and the constant concentration was 94 to 106 per cent (mean, 98 per cent) of that predicted if the injected dose were distributed into the Evans blue volume. It was concluded that HISA in small volumes was rapidly and almost completely absorbed from the injection site in the spleen and that its rate of appearance in the circulation could be determined if frequent samples were taken in the first 4 minutes. No significant leakage from the circulation occurred within the period of study.

Measurement of collateral blood flow was attempted in 12 patients after an overnight fast and without premedication. On the day prior to the test, the rate of removal from the blood of BSP (5 mg per kg) was determined. The volume of distribution (plasma volume), the rate of removal of BSP and the rate of constant infusion necessary for measurement of hepatic blood flow were calculated (7). Samples were analyzed for BSP by the technique of Seligson, Marino and Dodson (8).

On the morning of the test a no. 9 radiopaque catheter was introduced into a right hepatic vein and the wedged hepatic vein pressure recorded. All pressures were expressed in millimeters Hg above the measured right auricular pressure and were obtained with a Müller-Shillingford photoelectric transducer and recorder manufactured by the Cambridge Instrument Co. (9). Normal values for wedged hepatic vein pressure are less than 12 mm Hg (10–13).

An indwelling needle was inserted into a femoral artery and a priming dose of BSP, followed by a constant infusion of BSP, was administered into the arm vein. Paired samples of arterial and hepatic venous blood were withdrawn at intervals for determination of hepatic plasma flow (14).

Intrasplenic pressure was then measured (15) and, with the needle in position, 2 to 20 μc of HISA in 1 to 2 ml was injected deeply into the spleen. Normal intrasplenic pressure is less than 14 mm Hg (15, 16). Starting at the time of injection, continuous simultaneous manual withdrawal of blood was made from the hepatic vein catheter and from the femoral artery at the rate of 1 ml each 5 seconds. Syringes were usually changed at 30-second intervals during the first 1.5 minutes and at 1 minute intervals thereafter for 3 additional minutes. A single sample was taken from each site after 10 minutes. Samples were heparinized and BSP and radioactivity were measured in plasma. Counting was performed in a well-scintillation counter that measured 1.2×10^6 cpm per μc of iodine^{131}. Plasma volume was measured with Evans blue dye in 4 subjects. The volume of distribution for BSP was used in the remainder.

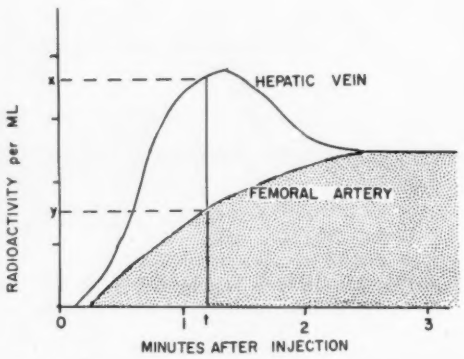

FIG. 2. HYPOTHETICAL RADIOACTIVITY OF FEMORAL ARTERIAL AND HEPATIC VENOUS PLASMA AFTER INJECTION OF HISA INTO SPLEEN AT ZERO TIME. Until absorption is complete, the hepatic venous values are greater than the femoral arterial values.

Calculation. A curve was constructed of the radioactivity measurements in the hepatic venous and femoral arterial plasma, such as is shown in the hypothetical example of Figure 2. The upper heavy line represents hepatic venous radioactivity and the lower line (at the upper border of the stippled area) represents femoral arterial radioactivity, which is assumed equal to the levels in recirculated arterial plasma leaving the splanchnic circulation at the same time. When the arterial and venous lines join, the absorption of radioactivity is complete and there is equilibrium.

Calculation is based on the premise that during the time required to reach equilibrium, the difference between the amount of radioactivity leaving the splanchnic bed and the amount entering it should be equal to the effective radioactivity injected minus the residual radioactivity in the splanchnic plasma. The radioactivity leaving the splanchnic bed is that leaving by the hepatic veins (Q_{hv}) plus that leaving by the collaterals (Q_{coll}). The radioactivity entering the splanchnic bed (Q_{fa}) is the product of the femoral arterial concentration and the hepatic plasma flow (F_{hv}). The effective radioactivity injected is the product of the equilibrium concentration (C_E) and the plasma volume (V_P). The residual splanchnic radioactivity is the product of the splanchnic plasma volume (V_{spl}) and the equilibrium concentration (C_E). The splanchnic bed is defined as those blood vessels that drain through the hepatic vein.

Then:

$$Q_{hv} + Q_{coll} - Q_{fa} = V_P C_E - V_{spl} C_E$$

$$Q_{coll} = (V_P - V_{spl}) C_E + Q_{fa} - Q_{hv}. \qquad [1]$$

TABLE I

Sample calculation

Time	Arterial	Venous	Difference	Area
min		*10^3 cpm/ml*		*(10^3 min)(cpm/ml)*
0–0.5	4.6	11.8	7.2	3.6
0.5–1.0	16.2	21.9	5.7	2.8
1.0–1.5	11.6	19.8	8.2	4.1
1.5–2.0	12.5	16.4	3.9	1.9
2.0–3.0	11.6	12.5	0.9	0.9
3.0–4.0	11.6	11.6		
				Total 13.3×10^3

In Equation 1, V_{spl} is assumed to be either 21 per cent of V_P or is calculated from Equation 1, assuming Q_{coll} to be zero. V_P and C_E are determined directly from the data. $Q_{fa} - Q_{hv}$ is obtained from the plot of the data as shown in Figure 2. In Figure 2, at any time (e.g., t) the vertical distance (x) to the upper line represents radioactivity per milliliter of plasma passing through the hepatic veins, and the vertical distance (y) to the lower line represents the portion of radioactivity derived from recirculated splanchnic arterial plasma. The distance (x − y) between the lines therefore represents the radioactivity per milliliter of hepatic vein plasma absorbed from the spleen. The radioactivity absorbed from the spleen via the hepatic veins between time t and time t + dt is therefore F_{hv} (x − y) dt microcuries. The total radioactivity absorbed through the hepatic veins or the unknown quantity $Q_{hv} - Q_{fa}$ in Equation 1 becomes

$$Q_{hv} - Q_{fa} = F_{hv} \int_0^T (x - y)dt. \qquad [2]$$

Sample calculation: A sample calculation (Table I) will better illustrate this manipulation. Table II contains pertinent clinical and experimental data.

The experimental hepatic venous and femoral arterial levels from Patient 1 are illustrated in Figure 3. Each vertical bar represents a single sample collected by continuous constant withdrawal of blood during the time indicated on the abscissa. $Q_{hv} - Q_{fa}$ is calculated from Equation 2. The value of the integral is obtained graphically by determining the area between the hepatic venous and the femoral arterial curves. Thus, in Figure 3 the value of the clear area for each block until equilibrium is listed.

This value when multiplied by the plasma flow (Table II) in milliliters per minute yields $Q_{hv} - Q_{fa}$ ($13.3 \times 10^3 \times 665 = 8.72 \times 10^6$). If the concentration in the femoral artery is greater than that in the hepatic vein the difference is calculated as zero; negative values are not considered. If Q_{coll} is *assumed* to be zero, the value of V_{spl} may be calculated directly from Equation 1. This value is recorded for each patient in Table II as the calculated V_{spl} expressed as a percentage of V_P.

For this patient the calculation is as follows:

Equation 1:

$$\frac{V_{spl}}{V_P} = \frac{Q_{fa} - Q_{hv} - Q_{coll} + V_P C_E}{V_P C_E}$$

$$= 1 - \frac{8.82 \times 10^6}{11.2 \times 10^6} = 0.22.$$

Thus the V_{spl} is 22 per cent of V_P.

In order to calculate collateral flow, V_{spl} is assumed to be 21 per cent of V_P, and Equation 1 is rearranged to yield:

Equation 1:

$$\frac{Q_{coll}}{Q_{coll} + Q_{hv} - Q_{fa}} = \frac{0.79 V_P C_E + Q_{fa} - Q_{hv}}{0.79 V_P C_E}$$

$$= 1 - \frac{8.72 \times 10^6}{8.84 \times 10^6} = 0.01.$$

Thus the fraction of portal blood flowing through collaterals is 1 per cent.

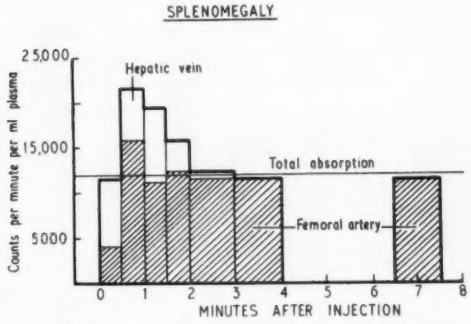

FIG. 3. PLASMA CONCENTRATION DATA FROM PATIENT 1. Each horizontal block is a single sample of plasma collected by continuous sampling during the time indicated by the widths of the block. Total absorption is calculated by dividing the dose given intrasplenically by the measured blood volume.

RESULTS

The clinical details, splenographic findings and calculation of collateral flow in 12 patients are given in Table II. Patients 1 to 3 had no clinical evidence of collateral blood flow. The calculated collateral blood flows are very low, in agreement with the clinical data. The calculated splanchnic plasma volumes of 22, 18 and 20 per cent of the total were in excellent agreement with values from the literature (17–19). Patient 4 had had ascites on admission but at the time of study the ascites had disappeared. There was no clinical evidence of collateral vessels, but portal hypertension was present. The calculated splanchnic plasma volume was 38 per cent and the calculated collateral flow was 22 per cent.

Patients 5 to 7 had cirrhosis with definite clinical evidence of collateral circulation. The calculated splanchnic plasma volumes were 36, 55 and 85 per cent of the plasma volume, respectively.

TABLE II

Clinical and experimental data on twelve patients with splenomegaly

Patient	Age	Sex	Diagnosis	Ascites*	Clinical collateral circulation			Estimated portal pressure		F_{hv}	$V_P \times C_E$	V_P	$\int_0^T (x-y)dt$	Calc.§ $V_{sp.}$	Collateral flow
					Varices	Abdo. wall	Venogram†	Wedged H.V.P.‡	Intrasplenic pressure						
								mm Hg	*mm Hg*	*ml/min*	*cpm*	*L.*	*mins cpm/ml*	%	%
1	28	M	Thalassemia minor	0‖	0	0	0	3	9	665	11.2×10^6¶	3.90	13.3×10^3	22	1.3
2	24	F	Thalassemia minor	0	0	0	**	2	6	1,030	3.02×10^6	2.54	2.40×10^3	18	0
3	52	F	Sarcoid of liver	0	0	0	0	11	14	1,065	1.64×10^6	3.21	1.23×10^3	20	0
4	54	M	Hodgkin's disease	1+	0	0	0	16	19	1,130	18.93×10^6	3.45	10.4×10^3	38	22
5	60	M	Cirrhosis	0	1+	1+	2+	19	17	491	2.11×10^6	3.90	2.75×10^3	36	19
6	35	F	Postnecrotic cirrhosis	0	1+	1+	**	16	**	645	0.802×10^6	5.06	0.56×10^3	55	43
7	45	M	Portal cirrhosis	2+	0	1+	3+	21	25	608	3.04×10^6	3.60	0.750×10^3	85	81
				1+	Side-to-side portacaval shunt			7	12	297	3.18×10^6	2.88	** ††	0	100
8‡‡	49	F	Portal cirrhosis	0	End-to-side portacaval shunt			6	10	434	14.4×10^6	3.70	**	5††	100
9	42	F	Portal cirrhosis	0	Side-to-side portacaval shunt			7	7	145	4.27×10^6	3.90	**	0	100
10	52	M	Portal cirrhosis	2+	2+	2+	2+	17	19	360	2.30×10^6	3.84	**	0	100
11	51	F	Portal cirrhosis	3+	3+	3+	3+	23	22	800	1.94×10^6	4.01	**	0	100
12	42	M	Myelofibrosis	0	0	0	**	7	10	1,115	1.42×10^6	4.33	**	0	100

* 1+ = History of ascites; 2+ and 3+, increasing degrees present.
† 0 = No collaterals; 1+, collateral vessels seen; 2+, one major collateral; 3+, two or more major collaterals.
‡ Wedged hepatic venous pressure.
§ Calculated splanchnic plasma volume as per cent total plasma volume.
‖ 0 = Determined but not present.
¶ This value corrected for previous HISA given for V_P determination.
** Not determined.
†† See text.
‡‡ Femoral vein instead of femoral artery.

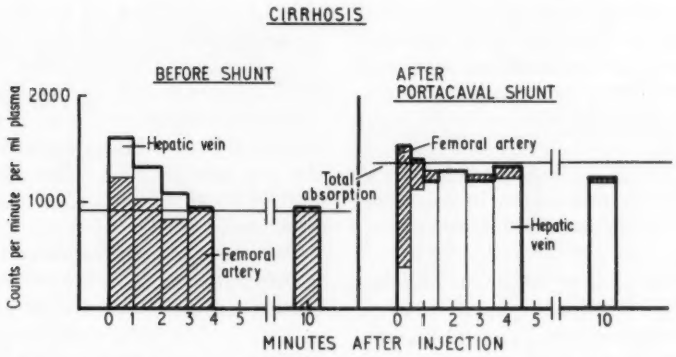

FIG. 4. TWO SIMILAR STUDIES ON PATIENT 7. In the first study the hepatic venous values were greater than those for the femoral artery. This was reversed after the shunt.

The corresponding calculated collateral flows were 19, 43 and 81 per cent. The results in a patient with collateral circulation (Patient 7) are illustrated in Figure 4; this patient was studied before and after side-to-side portacaval shunt. In the study before shunting, the hepatic venous samples prior to equilibrium contained more HISA than did the femoral arterial samples. The area between the curves was smaller than in Figure 3, indicating that a smaller proportion of the HISA was absorbed via the liver than in the patient without collaterals. The calculated collateral flow was 81 per cent.

In Patient 7, following a side-to-side portacaval shunt, the arterial blood contained more radioactivity than did the hepatic venous blood, indicating that radioactivity arrived at the liver first through the hepatic artery. Therefore, the calculated collateral flow was 100 per cent. Two other patients were studied after surgical portacaval shunts. In Patient 8 the shunt was end-to-side and in Patient 9, side-to-side; in both, the peripheral blood contained more radioactivity than did the hepatic venous blood until equilibrium was reached. The patients with past or present ascites had a higher calculated collateral flow than had those without ascites.

In Patients 10, 11 and 12 the results were quite unexpected. Both 10 and 11 had cirrhosis with ascites and clinical evidence of marked collateral circulation. In both, the radioactivity in the arterial blood was higher than that in the hepatic venous blood. The peculiar finding was also present in Patient 12 who had no clinical evidence of collateral circulation.

DISCUSSION

The important assumptions used in calculating the collateral flow are listed in the Method section. Several of these deserve comment.

The absorbed HISA must mix completely in the splenic vein before the first effluent venous branch is reached and at all points where new blood is added. Though mixing is probably complete in the small vessels and in splenic and portal veins of normal size, the increased diameter of the vessels and the reduced blood flow in portal hypertension make streamlining possible and even probable (20). This has been frequently observed in splenic venograms, particularly at the entrance of the inferior and superior mesenteric veins (16, 21).

The splanchnic plasma volume cannot be measured in the presence of collateral circulation (17); it must therefore be estimated in order to calculate the collateral flow. The value has been determined in subjects without liver disease. A mean splanchnic plasma volume of 21 per cent of the plasma volume was found (17–19); the variation, however, was large. The portal vein and its branches are dilated in portal hypertension; their capacity is therefore enlarged. In the presence of a collateral circulation, part of the portal vein blood drains through the liver and part drains through the collateral vessels. The splanchnic plasma volume used in Equation 1 is defined as

the volume of plasma contained in the vessels draining through the liver. By this definition the fraction of splanchnic blood in the collaterals must not be included. Even though the capacity of the portal bed is enlarged, the value of 21 per cent is still reasonable.

The radioactivity in the femoral artery does not exactly represent the radioactivity in recirculated arterial blood. Recirculated radioactivity mixed in the splanchnic bed, and sampled as hepatic venous blood, left the aorta some time earlier (about 12 seconds) than the blood sampled simultaneously at the femoral artery. In portal hypertension the circulation time of blood from the aorta to the hepatic veins is prolonged and therefore this error is greater. This prolongation in portal hypertension is approximately 20 seconds (20, 22–24). Applying this correction to the data decreases the calculated collateral flow by an average of about 8 per cent. This error assumes particular importance if some radioactivity reaches the peripheral circulation through a collateral vessel *much* earlier than it would through the liver, possibly by-passing the pulmonary circulation. Under these circumstances the radioactivity in the peripheral arterial blood is much higher than the simultaneously sampled recirculated arterial blood mixed in the portal bed and sampled at the hepatic vein. Such an error leads to overestimation of the collateral flow.

A change in blood flow of 5 to 10 per cent following the intrasplenic injection might be missed in the short time of sampling by the BSP technique.

In Patients 1 to 3 the calculated splanchnic plasma volumes were in very good agreement with the values given in the literature. In Patients 4 and 5 the calculated splanchnic plasma volumes of 36 and 38 per cent were just outside the range of normal in patients without collaterals. Therefore, such values may represent the splanchnic plasma volume. On the other hand, both patients showed clinical evidence of collateral vessels, suggesting that calculated V_{spl} is too high.

In Patients 6 and 7 the calculated values of 55 and 85 per cent for the splanchnic plasma volume are obtained, assuming no collateral flow. Such high values for splanchnic plasma volume are extremely unlikely; collateral flow must be present.

In patients with end-to-side portacaval shunts, radioactive blood reaches the liver only through the hepatic artery, and all of this blood leaves the liver through the hepatic veins without bypass. The volume of blood in all vessels from the hepatic artery to the hepatic veins may be calculated (17). In Patient 8 the value for this plasma volume of 5 per cent was obtained. This agrees adequately with the figure of 6 per cent for this plasma volume found in dogs (25).

Patients 10 to 12 had calculated collateral flows of 100 per cent. This is unexpected in patients without surgical portacaval shunts. In all three patients the radioactivity was higher in the peripheral artery than in the hepatic vein. The highest values of radioactivity in the peripheral artery in these patients appeared in the first half minute after sampling (as in Figure 4 after shunt). The highest values of radioactivity in the artery in all other patients appeared later, just after the end of the first minute on the average. The rate of appearance of radioactivity in the hepatic venous blood was about the same as the rate in the other patients. These findings suggest a high blood flow of short circulation time through collateral vessels. Such a shunt has been observed by Reichman, Davis, Storaasli and Gorlin (26) and may represent the shunt from the portal vein to the left side of the heart demonstrated in the anatomical studies in cirrhotic patients of Abelmann and coworkers (27). Such a mechanism would produce a high concentration of HISA in a very small part of the peripheral circulation (left heart and aorta) before this portion of radioactive material could appear in the hepatic vein by recirculation. Patient 11 showed oxygen-refractory desaturation of the arterial blood which is evidence that venous blood bypasses the lung.

Another explanation of these findings would be a misplaced catheter in the inferior vena cava. The removal of BSP, however, showed that the catheter remained in the hepatic vein. A further explanation could be the injection of HISA into the muscle or peritoneal cavity. The recording of the intrasplenic pressure in all three patients and the nearly complete absorption of injected HISA in two of the three indicates that the needle was in the spleen. In Patient 12, however, only one-third of the administered dose appeared in the circulation in ten minutes. Equilibrium was reached in three minutes. Megakaryocytes were

seen in a smear made from the needle just prior to injection, confirming that the needle was in the spleen. There is no satisfactory explanation of the findings in Patient 12.

SUMMARY

A method for the estimation of blood flow through the collateral vessels of the portal vein is described. The concentration of iodinated albumin injected into the spleen appearing at the hepatic vein and in the peripheral circulation is measured, and from the difference the collateral flow is estimated. The results of this method correlate with clinical and radiological evidence of collateral circulation. Some patients show very rapid circulation of the iodinated albumin through collateral vessels which makes application of the method impossible.

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BEHAVIOR OF STROKE VOLUME AT REST AND DURING EXERCISE IN HUMAN BEINGS *

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The implications of Starling's law of the heart, as stated in the Linacre Lecture (1), make it clear that physical exercise brings about increased venous return and increased diastolic filling of the heart. This, in terms of the Starling concept, induces sufficient increase in the energy of contraction to drive increased volumes of blood into the aorta with each stroke. While Starling was aware that "the heart is subject to the control of the central nervous system by means of its inhibitor and augmentor nerves . . ." he undoubtedly placed the emphasis, insofar as cardiac adaptation to stress is concerned, on intrinsic response of the myocardium to change in end-diastolic fiber length. It follows, if Starling's Linacre Lecture is credited, that the heart must meet the demands imposed by physical exertion, in part at least, by increasing its stroke volume.

This was indeed found to be the case in older studies on the effect of exercise on human circulatory function (2–4); these studies served further to establish the view that increase in both stroke volume and pulse rate are utilized by the organism during exercise.

A different view has been gradually formulated by Rushmer who, using continuous measurements of internal left ventricular diameters (5) and circumference (6) in unanesthetized dogs exercising on a treadmill, was not convinced that response to such stress was entirely in accord with Starling's law. These results, and an examination of other workers' data on human subjects, ultimately led him to a radical revision of the older view concerning the interdependence of increase in stroke volume, pulse rate and arteriovenous oxygen difference in adaptation to exercise. In his words (7): "Evidence that the stroke volume need not change significantly during exercise which increases oxygen consumption by more than 10-fold has been accumulating in the literature during the last 20 years." In his most recent comment on the point, Rushmer modified his earlier views somewhat by invoking postural effects on stroke volume to explain the large amount of conflicting data in the literature, but he still holds that "the stroke volume increases but little during exertion and rarely exceeds recumbent control values" (8).

Because data on human subjects from our laboratory were in conflict with Rushmer's conclusions, it was decided to re-examine our results and to add new studies to fill in the gap between the resting state and very heavy exercise loads.

METHOD

Studies on 26 normal men, aged 19 to 63 (average 28) form the basis for this report. Data on 15 of the subjects were previously reported in studies on maximal oxygen intake (9, 10). Since the previous experiments were designed to yield information only under resting conditions (subject standing) and at exercise loads producing maximal oxygen intake, 11 additional subjects were tested under different conditions. In 5 subjects, initial measurements were made with the subject supine and at rest. Immediately following, the measurements were repeated with the subject standing at rest. Further measurements were then made at very low levels of exercise. In the other 6 subjects the procedure was the same except that no studies were done with the subject supine.

The experimental procedure was the same as that previously reported (9). Brachial venous and arterial catheters were inserted into the left arm. For measurement of cardiac output, 10 mg of Evans blue (T-1824) was delivered at the end of the catheter into the brachial vein with the subject at rest or, in the exercise studies, 1.5 minutes after beginning of exercise (during the collection of expired air). Arterial samples were collected at 1 second intervals in tubes containing powdered heparin, beginning at the time of injection and continuing for 40 seconds. Analysis of blood samples for dye content was carried out using a Beckman model DU spectrophotometer. The data were then plotted and extrapolated by the method of Hamilton, Moore, Kinsman, and Spurling (11). Such error as the sampling technique may in-

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troduce, especially at heavy work loads, is probably not a systematic one insofar as measurement of cardiac output is concerned.

Pulse rate and oxygen intake (expired air method) were measured over a period including that used for the determination of the cardiac output. After resting determinations were completed, the subject performed on a motor-driven treadmill for 2.5 minutes. Oxygen intake, cardiac output and pulse rate were measured during the last minute of the exercise period. Grades and speeds were set to provide increasing levels of exercise. In most subjects no more than 3 exercise periods, spaced at least 15 minutes apart, were used.

Mean stroke volume was calculated by dividing the cardiac output by pulse rate. Arteriovenous oxygen difference was derived from cardiac output and oxygen intake by use of the Fick equation.

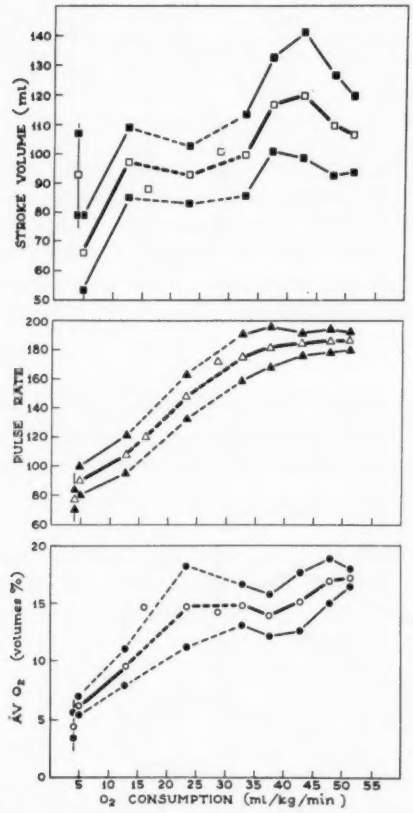

FIG. 1. STROKE VOLUME, PULSE RATE AND ARTERIOVENOUS OXYGEN DIFFERENCE WITH INCREASING LOADS OF EXERCISE. Data are tabulated in Table I. Supine resting values are at extreme left, connected by a vertical line. In each of the three plots, the central heavy line is the mean, the two outer lines representing one standard deviation above and below the mean. The dashed lines represent portions of the oxygen intake scale for which data are numerically inadequate (Table I). For example, at oxygen intake of 15 to 19.9 ml per kg per minute, only one value is available.

RESULTS

The data are set out in Table I and in Figures 1, 2 and 3.

For plotting, the data were grouped according to oxygen intake in milliliters per kilogram per minute, in 4.9 ml increments. In Figure 1 the mean oxygen intake for each group is plotted on the abscissa; the mean stroke volume in milliliters is plotted on the ordinate. Plotting stroke index in milliliters against oxygen intake expressed as liters per minute per square meter body surface yielded curves that were almost identical with those in Figure 1.

In Figure 2, data on four subjects are plotted in order to give some idea of individual variation in the response of stroke volume to increasing levels of exercise.

In Figure 3, a plot recently published by Rushmer (8) is the basis for the upper curve. His first plot of this type (7) was based on data obtained from seven groups of investigators who used the direct Fick or the dye dilution method for determining cardiac output during exercise. Subsequently he added data supplied by us, some of which were published in the paper by Mitchell, Sproule and Chapman (9), to obtain the plot used for construction of the upper curve in Figure 3. For present purposes, the plot was photographically enlarged and covered with a grid so that the values not otherwise available to us could be read off. Owing to the heavy grouping of the data at oxygen intakes below 500 ml per minute, the initial mean value used in our replot is an estimate. The other points on the replot are mean figures obtained by grouping individual stroke volume values for each 500 ml increment of oxygen intake (Table II). The second curve (labeled observed data) is the same as that plotted in Figure 1 except that the values on the abscissa are expressed in liters of oxygen per minute (instead of in milliliters of oxygen per kilogram per minute). It should be apparent, therefore, that the two upper curves are, in part, based on the same data. This is especially

TABLE I

Mean values at rest (supine and standing) and at increasing levels of oxygen intake of 26 normal men (average age 28)

Group (O_2 intake)		No.	Mean O_2 intake	Cardiac output	Pulse rate	Stroke vol.	AVO_2 diff.	Surface area
ml/kg/min Range	Mean		*L/min*	*L/min*		*ml*	*ml/100 ml*	*sq m*
Supine rest	3.9	5	0.304±0.034	7.2±1.5	77± 7	93±14	4.4±1.4	1.96±0.14
Standing rest	4.8	16	0.346±0.052	5.8±1.3	90±10	66±13	6.1±0.7	1.91±0.18
10–14.9	12.7	6	0.948±0.108	10.2±1.1	108±13	97±12	9.4±1.5	1.95±0.12
15–19.9	16.1	1	1.526	10.5	120	88	14.5	2.16
20–24.9	23.1	3	1.940±0.334	13.7±1.2	148±15	93±10	14.5±3.5	2.05±0.19
25–29.9	28.4	1	2.448	17.3	172	101	14.2	2.12
30–34.9	32.7	6	2.502±0.273	17.6±3.2	175±16	100±14	14.6±1.8	1.98±0.16
35–39.9	37.4	11	2.885±0.336	21.2±3.6	182±14	117±16	13.9±1.8	1.98±0.15
40–44.9	42.7	12	3.222±0.496	21.9±4.3	184± 8	120±21	15.1±2.4	1.95±0.21
45–49.9	47.8	7	3.401±0.602	20.5±4.0	186± 8	110±17	16.9±1.9	1.91±0.19
50+	51.1	3	3.380±0.463	19.9±3.3	186± 6	107±13	17.1±0.7	1.82±0.16

true of the portions depicting behavior of stroke volume at high oxygen intakes.

Results of tests of significance between points on the stroke volume curve in Figure 1 are given in Table III. They show that the resting supine value is significantly higher than the value obtained during standing rest. Both resting values (supine and standing) are significantly lower than the two highest values (117 and 120 ml). The standing resting value is significantly lower than all those obtained during exercise. In applying tests for significance to the replot of Rushmer's collected data, it was not possible to utilize the first value (resting) on the curve because the number of observations was not known. Comparison of the second value (96 ± 13 ml) with the other points on the curve shows it to be significantly lower than the last three points.

DISCUSSION

The present study demonstrates quite clearly that stroke volume increases with increasing levels of exercise although the relation is probably not

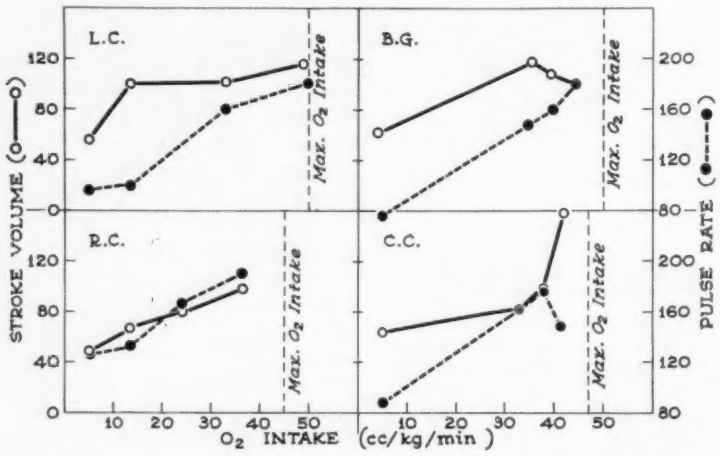

FIG. 2. RELATION OF STROKE VOLUME (SOLID LINE) AND PULSE RATE (BROKEN LINE) TO OXYGEN INTAKE IN FOUR INDIVIDUAL SUBJECTS. The first point (extreme left) is the standing resting value. Subsequent values were obtained at increasing exercise loads.

TABLE II

*Mean values obtained from the plot of collected data by Rushmer (8)**

Group (O_2 intake)	No.	Mean O_2 intake	Stroke volume
L/min		*L/min*	*ml*
0.5–1.0	54	0.730 ± 0.140	96 ± 13
1.0–1.5	29	1.202 ± 0.108	108 ± 25
1.5–2.0	23	1.728 ± 0.138	113 ± 34
2.0–2.5	17	2.230 ± 0.187	98 ± 21
2.5–3.0	19	2.776 ± 0.140	114 ± 32*
3.0–3.5	17	3.187 ± 0.138	132 ± 25*
3.5–4.0	8	3.722 ± 0.175	136 ± 31*

* Significant difference between values marked and initial value (96 ± 13); p values are <0.05, <0.001 and <0.01, respectively.

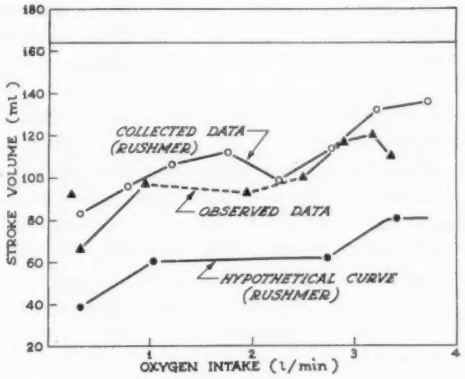

FIG. 3. COMPARISON OF STROKE VOLUME CURVES WITH INCREASING EXERCISE LOADS. Upper and lower curves were replotted from a publication by Rushmer (8). Data for upper curve given in Table II.

a linear one. The conclusion is true as stated whether one uses standing or supine resting conditions as a starting point. It would appear, in fact, that from one-third to about one-half of the increase in cardiac output near the maximal oxygen intake level (21.9 L per minute) over the resting (7.2 L per minute) is attributable to increase in stroke volume, depending on whether one uses the resting supine or resting standing value for comparison. If the value for stroke volume prevailing under resting supine conditions also obtained during heavy exercise, when the pulse rate was 184, cardiac output would have been 17.1 L per minute. The difference between the observed cardiac output near the maximal oxygen intake level and the theoretical value is 4.8 L per minute, which represents about one-third of the observed increase (14.7 L per minute). Using the resting standing value for stroke volume for comparison, the influence of increase in stroke volume upon cardiac output during heavy exercise is much more striking.

Careful inspection of the two plots published by Rushmer suggests that a definite upward trend is present in the second plot (8) (which includes our data); there may also be a slight upward trend in the lefthand portion of the first plot (7). It should be noted also that, when the data are regrouped as in Figure 3, stroke volume at high levels of exercise rises significantly above resting values, which may well include both supine and standing data. The intermediate portion of the stroke volume curve is still not convincingly determined; at such levels of exercise, stroke volume may possibly rise very slowly or may remain more or less constant.

TABLE III

*Test of significance applied to data in Table I and Figure 1 (top)**

Comparison	Mean values	p
Resting supine vs. resting standing	93 vs. 66	Less than 0.01
Resting supine vs. value at 35–40 ml/kg/min	93 vs. 117	Less than 0.05
Resting supine vs. value at 40–45 ml/kg/min	93 vs. 120	Less than 0.02
Resting supine vs. value at 45–50 ml/kg/min	93 vs. 110	Not significant
Resting standing vs. value at 10–15 ml/kg/min	66 vs. 97	Less than 0.01
Resting standing vs. value at 20–25 ml/kg/min	66 vs. 93	Less than 0.01
Resting standing vs. value at 30–35 ml/kg/min	66 vs. 100	Less than 0.001
Resting standing vs. value at 35–40 ml/kg/min	66 vs. 117	Less than 0.001
Resting standing vs. value at 40–45 ml/kg/min	66 vs. 120	Less than 0.001
Resting standing vs. value at 45–50 ml/kg/min	66 vs. 110	Less than 0.001
Resting standing vs. value at 50+ ml/kg/min	66 vs. 107	Less than 0.01

* $t = m_1 - m_2/\sqrt{(SEm_1)^2 + (SEm_2)^2}$.

The actual situation in normal subjects who are neither highly trained nor habitually inactive seems, therefore, to be similar to that suggested in Rushmer's hypothetical curve (Figure 3) except that the values for stroke volume it represents are too low. None of the curves is consonant with the view that stroke volume increases very little during exertion and rarely exceeds recumbent control values even at heavy exercise loads in which oxygen intake is about ten times the resting intake. As it stands, the statement is applicable only to the situation prevailing in mild exercise. One might question its general validity even in this case since, in human subjects, exertion often follows periods of quiet standing when stroke volume is unquestionably at its lowest. As for heavy exercise, such as that producing maximal oxygen intake, it seems to be impossible for normal human subjects to make the necessary adaptation *without* increasing stroke volume. Thus, although Rushmer's original rejection of "generally accepted dogma" (7) appears to have been too sweeping, a resolution of the difficulty is reached by allowing for the influence of position on resting stroke volume, *vis-a-vis* those prevailing at low levels of exercise, and by noting the impressive extent to which stroke volume is utilized at high levels.

The matter is, in fact, of some theoretical importance. If it could be shown that adaptation to exercise over the full physiologic range can be accomplished solely, or even primarily, by increase in pulse rate, the significance of changes in intrinsic myocardial reactivity would be doubtful. That intrinsic myocardial response changes during exercise has been demonstrated (12), although it has not been possible to study the phenomenon quantitatively at high exercise loads. The role of the Starling mechanism in adaptation to exercise thus again comes into focus. It is a question, in a sense, of what Starling actually meant by the law bearing his name. If one insists that he considered venous return to be the vital initiating factor in cardiac response to exercise, and the keystone in his whole theoretical arch, then to an extent Startling's law may be invalid. If, on the other hand, one considers that the fundamental feature of the law, as set out by Frank (13), Straub (14) and Patterson and Starling (15), is a relationship between "force of contraction," or stroke work, and end-diastolic volume, evidence now available indicates that the principle plays a definite role in cardiac response to exercise. The increase in stroke volume seen with increasing loads of exercise is a manifestation of this role and would be difficult to explain if some such principle were not operative.

A complete understanding of the cardiovascular response to exercise is by no means at hand. Obviously, individual differences in response are considerable, as suggested by Figure 2. Factors determining what mechanism will be dominant, especially at low to moderate levels of exercise, are not known. Very probably, physical training, age and body composition are involved. There can be little doubt, however, that in higher organisms, including man, adaptation to exercise requires an intricate interdigitation of neural, myocardial and chemical factors and that ability to increase stroke volume is one of the mechanisms involved.

CONCLUSIONS

1. Increase in stroke volume is one of the mechanisms normally utilized in cardiovascular adaptation to exercise in man.

2. The relative importance of the mechanism appears to be greatest at the transition from rest (subject standing) to mild exercise and at levels of exercise approaching those producing maximal oxygen intake.

3. The highest levels of stroke volume reached during heavy exercise are significantly greater than those prevailing at rest, whether the subject is supine or standing.

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CONJUGATION OF SULFOBROMOPHTHALEIN SODIUM WITH GLUTATHIONE IN THIOETHER LINKAGE BY THE RAT *

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Most of the sulfobromophthalein sodium (BSP) appearing in the bile of man, rat, dog, sheep and cat differs chromatographically from the free BSP originally administered (1–4). The altered BSP compounds in man, rat and dog appear to be amino acid conjugates (2–4). Thus, after hydrolysis of the major BSP compounds with 6 N HCl, several amino acids have been detected by filter paper chromatography. Glycine, glutamic acid and alanine were identified in man (2, 3), glycine and glutamic acid in the rat (2) and glycine in the dog (3).

Recently, in a preliminary report, Javitt, Wheeler, Baker and Ramos (5) suggested that BSP appears in bile of the dog as a conjugate of glutathione. These authors found glycine, glutamic acid and evidence of free sulfhydryl groups, as manifested by a positive sodium azide-iodine reaction, after alkaline hydrolysis of the major BSP conjugates. Occasionally, cysteine was detectable. BSP conjugates formed *in vitro* by admixture of BSP and glutathione were identical electrophoretically and chromatographically with conjugates found in bile. Cysteine has also been identified in human BSP conjugates by Grodsky, Carbone and Fanska (4). Cysteine was difficult to detect after hydrolysis with concentrated HCl, but was more clearly demonstrated when the BSP compounds were hydrolyzed with HBr. Glycine and glutamic acid were also detected after hydrolysis of the BSP conjugates. However, these amino acids were found in hydrolysates of control bile processed in the same fashion as BSP bile. Therefore, these authors regarded glycine and glutamic acid as possible contaminants of the BSP metabolites.

In the present report, evidence will be presented that BSP is conjugated with glutathione in the rat. Furthermore, it will be demonstrated that the linkage of glutathione to BSP occurs through the sulfhydryl group of cysteine.

MATERIALS AND METHODS

Four to 5 mg of S^{35}-labeled reduced glutathione [1] which contained 20 to 30 μc of radioactive sulfur was injected intraperitoneally into 7 rats. Two rats received approximately 5 mg of S^{35}-labeled L-cystine [1] containing 25 μc of radioactivity. Ten μc of inorganic $S^{35}O_4$ in normal saline was administered to an additional 2 rats. Two to 4 hours later, polyethylene tubing was inserted into the common bile duct and a control bile sample was collected for 45 minutes. BSP in amounts of 4 to 5 mg per 100 g body weight was then injected intravenously and additional bile was collected over the ensuing 45 minutes. In four experiments, S^{35}-labeled BSP [2] was given intravenously and bile was collected for 3 hours after the common bile duct was intubated.

Descending chromatograms of bile were made on Whatman no. 1 filter paper strips. The distribution of radioactivity and of BSP on the chromatograms was then determined. The chromatographic techniques and the methods of quantitating radioactivity and BSP color on chromatograms have been described in detail in a previous publication (2). Briefly, two adjacent strips 1 to 1.5 cm wide were cut longitudinally from the chromatograms. After sectioning at 1 cm intervals, radioactivity was measured on one strip by placing each segment of paper in a counting bottle containing a phosphor and counting in a liquid scintillation counter. The distribution of BSP on the adjacent strip was determined by eluting the BSP contained in each segment of paper into 0.1 N potassium hydroxide and measuring the optical density of the eluate in a Beckman DU spectrophotometer set at 575 mμ. Where the ratio of counts to BSP color in a single BSP compound was determined before and after passing the compound through an ion exchange

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† Established Investigator of the American Heart Association.

[1] Schwarz Laboratories, Inc., Mount Vernon, N. Y.

[2] Volk Radiochemical Co., Chicago, Ill.

column or acid hydrolysis, duplicate chromatograms of the BSP compound were developed. Total radioactivity contained in the BSP spot on one chromatogram and BSP color contained on the duplicate were determined.

To prepare BSP compounds for chemical analysis and amino acid chromatography, BSP bile was pooled from several rats. The BSP compounds were separated by descending chromatography as previously described (2). The BSP-containing bands were located on the paper by exposure to ammonia vapors and eluted into water. The eluate was passed through the Dowex 50-×8 200 to 400 mesh ion exchange column to remove any free amino acids or peptides that migrate with the same R_f as the BSP compounds. The resulting solutions were concentrated under a stream of nitrogen in a 60° C water bath.

Conjugation of the lactone ring in the BSP compounds with amino acids to form a peptide bond was tested for by examining the capacity of the compounds to form hydroxamic acids. One ml of the concentrated BSP solutions was evaporated to dryness in a water bath at 60° C and the procedure outlined by Bauer and Hirsch (6) was followed. BSP standards were run simultaneously with the BSP compounds separated from bile.

The reactions with hydroxylamine and nitroprusside on paper as described by Stadtman (7) were used to determine the presence of a thioester linkage in the major BSP conjugate. The reactions of S-acetyl glutathione, a known thioester, and free BSP were also examined.

The presence of a thioether bond in the major BSP conjugate was tested for by the iodometric method of Stekol (8). An excess of iodine was added to the solution and the quantity of iodine consumed was determined by back titration with standard thiosulfate solution. The molarity of the iodine and thiosulfate solutions used was 0.0005 M since very small quantities of the BSP conjugate were available for analysis. Even at these low concentrations, recovery of mercaptoethanol, a known SH compound, was satisfactory. Thus, when 1 ml of a 5×10^{-4} M solution of mercaptoethanol was analyzed directly for its SH content, the recovery was 108 per cent (Table VI).

The sodium azide-iodine reaction was performed as a spot test as outlined by Feigl (9). One drop of the test solution was mixed with one drop of sodium azide-iodine solution. The time at which bubbles were first detected and the character of the bubbles were noted.

To identify the amino acid composition of the BSP compounds, the conjugates were hydrolyzed in 5.9 N HCl in sealed glass tubes for 16 to 18 hours at 110° C. Aliquots were pipetted onto a watch glass and brought to dryness under a stream of hot air. After redissolving in water and redrying to get rid of traces of HCl, the material was subjected to two-dimensional amino acid chromatography on filter paper (10, 11). Standard amino acids as well as hydrolyzed samples of glutathione were chromatographed simultaneously.

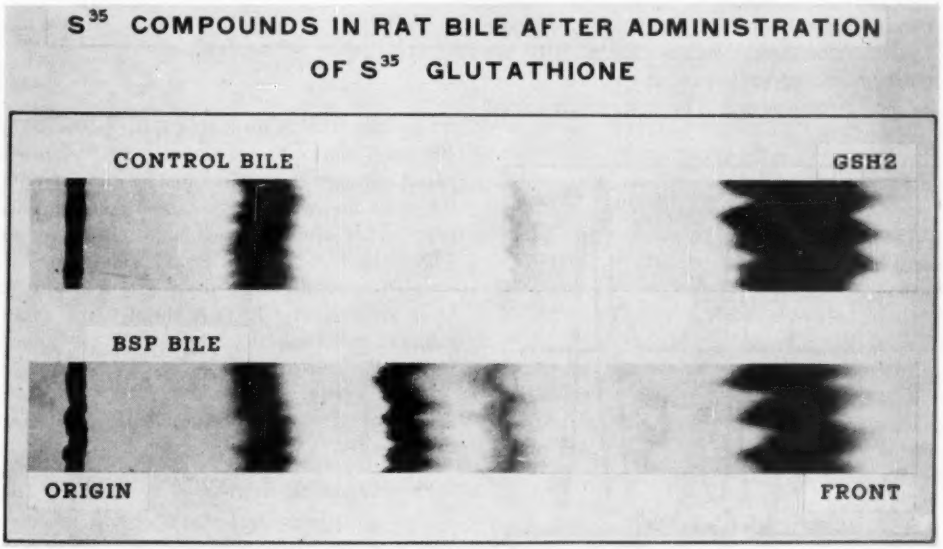

FIG. 1. RADIOAUTOGRAPHS OF CHROMATOGRAMS OF CONTROL BILE AND BSP BILE IN RAT GSH 2. Control bile was obtained after S^{35}-labeled glutathione was administered intraperitoneally. BSP was then injected intravenously and a BSP bile sample collected. Equal volumes of control and BSP bile were chromatographed on Whatman no. 1 filter paper strips in a descending system consisting of glacial acetic acid: water: *n*-propyl alcohol (1:5:10, vol/vol). The papers were dried and radioautographs developed.

RESULTS

1. Incorporation of radioactivity from S^{35}-labeled glutathione and S^{35}-labeled cystine into BSP conjugates

Radioactivity was associated with the BSP conjugates identified on chromatograms of bile collected from rats receiving S^{35}-labeled glutathione and S^{35}-labeled cystine. A representative radioautograph of such chromatograms from a rat receiving radioactive glutathione is demonstrated in Figure 1. Radioactive material was present in control bile obtained before BSP was administered. Similar radioactive bands were also detected in bile collected after BSP was injected. In addition, however, a very dense band, not detected in control bile, is seen in BSP bile. Other bands are seen in BSP bile, but it is difficult to be sure on the basis of such radioautographs whether they represent a qualitative or quantitative difference between control and BSP bile, since faint bands in similar location are seen in control bile.

The distribution of BSP and of radioactivity on the chromatogram from which the radioautograph in Figure 1 was made can be seen in Figure 2. Values for optical density due to BSP are plotted on the upper half of the figure. Three BSP peaks are evident. The first, which has its peak at 14 cm from the origin of the chromatograph, corresponds to BSP A, the major BSP conjugate in rat bile. The second has its peak 18 cm from the origin and corresponds to BSP B, another BSP conjugate. The third peak is at 27 cm and is composed of BSP D, which has been shown to be free BSP. The S^{35} in control and BSP biles is plotted on the lower half of Figure 2. It is evident that peaks of radioactivity which conform in shape and position with the BSP conjugates, BSP A and BSP B, are present only in BSP bile. The BSP conjugates in all of the animals receiving S^{35}-labeled glutathione and S^{35}-labeled cystine were labeled. By contrast, the free BSP excreted in bile did not contain radioactivity.

The ratio of counts per minute per unit optical density in BSP A was determined before and after passing BSP A through a Dowex-50× 200 to 400 mesh ion exchange column. BSP A readily passes through the column and is not altered by the procedure, whereas free amino acids and small peptides are bound to the column. It can be seen (Table I) that the quantity of radioactivity associated with the BSP conjugate remains the same after passing BSP A through the Dowex column, indicating that the S^{35} is an intimate part of the BSP conjugate.

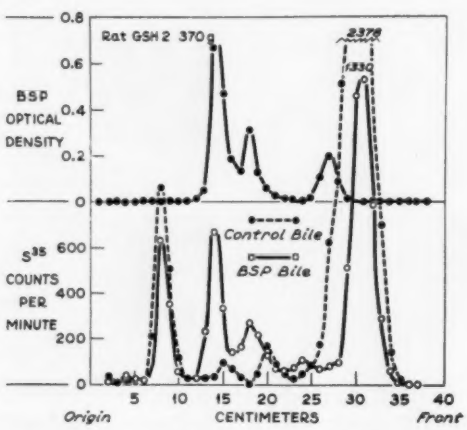

FIG. 2. THE DISTRIBUTION OF RADIOACTIVITY AND OF BSP ON THE CHROMATOGRAMS OF RAT GSH 2. The radioautographs of these chromatograms are shown in Figure 1. See text for description.

TABLE I

Relationship of S^{35} counts to BSP optical density in BSP A obtained from rats receiving S^{35}-labeled glutathione

Rat	Not through Dowex-50×	Through Dowex-50×
no.	*cpm/U optical density*	
GSH 4	1,724	1,798
GSH 7	1,328	1,303
GSH 8	973	947

2. Site of S^{35} incorporation into the BSP molecule in BSP conjugates

Several possible alternatives by which S^{35} from labeled glutathione and cystine could become a part of the BSP conjugates were examined.

a) Exchange of sulfur in the sulfonic acid groups. This exchange should occur only in the BSP conjugates, since the S^{35} label of glutathione was introduced only into the BSP conjugates and

TABLE II

Comparison of S^{35} counts to optical density in BSP A and BSP D after administration of $BS^{35}P$

Experiment	BSP A	BSP D
no.	*cpm/U optical density*	
1	49,400	48,600
2	69,300	72,100
3	47,500	50,100
4	51,700	51,400

not into free BSP. Thus, when BSP labeled with S^{35} in the sulfonic acid group is administered, sulfur exchange in the conjugates should lead to a lowering of their specific activity as compared with free BSP. The ratio of counts per minute per unit optical density due to BSP in BSP A and BSP D was determined in four experiments (Table II). The specific activities of the major BSP conjugate and free BSP did not differ significantly. Additional evidence against sulfur exchange is provided by the inorganic $S^{35}O_4$ experiments, in which no evidence was found for incorporation of radioactivity into the BSP compounds (Figure 3).

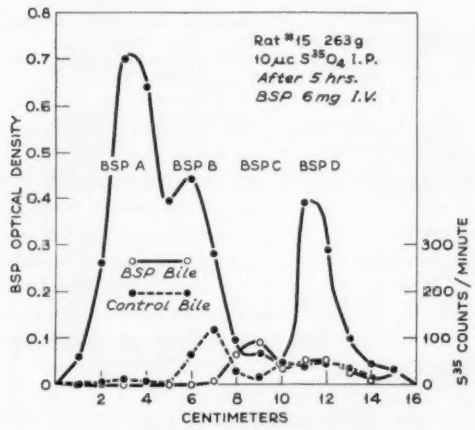

FIG. 3. THE DISTRIBUTION OF RADIOACTIVITY AND OF BSP ON THE CHROMATOGRAMS OF A RAT RECEIVING INORGANIC $S^{35}O_4$. Only that segment of the chromatograms containing the BSP bands is shown. The values for BSP optical density are indicated by the solid line with solid circles. The S^{35} counts per minute contained in BSP and control bile are shown by the solid line with open circles and the dashed line with solid circles, respectively. No incorporation of radioactivity into the BSP compounds is demonstrated.

b) Formation of an ethereal sulfate. The ratio of counts per minute per unit optical density in BSP A obtained from rats given S^{35}-labeled glutathione was determined before and after heating a solution of BSP A at 100° C for 30 minutes in 1 N HCl (Table III). Ethereal sulfates are known to be hydrolyzed under these conditions (12). No decrease in specific activity of the BSP conjugate was detected after hydrolysis in weak acid.

As a further test for the presence of an ethereal sulfate in the BSP compounds, radioactive inorganic sulfate was administered to two rats to see whether the radioactivity would appear in the BSP conjugates. This was carried out, since Isselbacher and McCarthy demonstrated radioactive label in the ethereal sulfate of bilirubin collected in bile when inorganic $S^{35}O_4$ was administered intraperitoneally to rats (13). In the two rats given inorganic $S^{35}O_4$ and then BSP, no radioactivity appeared in the BSP compounds recovered in bile. The distribution of BSP and of radioactivity on the chromatograms of one such animal is shown in Figure 3.

TABLE III

Effect of heating BSP A from rat receiving S^{35}-labeled glutathione, at 100° C for 30 minutes in 1 N HCl

	Counts per minute/U optical density	
Before heating	300	298
After heating	296	288

c) Conjugation with the lactone ring of BSP. Two types of linkage with the lactone ring of BSP might exist, namely peptide and thioester. The hydroxylamine reaction was used to test for the presence of a peptide bond in the BSP conjugates. If the lactone ring were engaged in peptide linkage, the BSP conjugates would not form hydroxamic acids. It was observed that free BSP (BSP D), BSP A and BSP C, obtained from bile pooled from several rats, all formed hydroxamic acids (Table IV). The ratio of found optical density to that in free BSP was 1.20 for BSP A and 1.18 for BSP C. Such deviations from unity are within the range of experimental error of the method. Although these findings ruled out the participation of the lactone ring with amino acids in peptide linkage, they did not ex-

TABLE IV

Reaction of BSP compounds with hydroxylamine

Compound	Concentration of BSP	Optical density of BSP-hydroxamic acid-iron complex		
		Found	Expected	Found/expected
	mg %			
BSP A	55.6	0.295	0.245	1.20
BSP C	23.0	0.120	0.102	1.18
BSP D (free BSP)	50.8	0.224		

clude a thioester bond, for thioesters have been shown to react with hydroxylamine to form hydroxamic acids (7). Therefore, the reaction of BSP A with hydroxylamine on paper (7) was carried out. A free sulfhydryl group should be identified after reaction of a thioester with hydroxylamine. Free BSP, S-acetyl glutathione, a mixture of these two, and BSP A formed hydroxamic acids after treatment with hydroxylamine, as evidenced by a positive reaction with ferric chloride reagent (Table V). Whereas none of the compounds exhibited a positive reaction for free sulfhydryl groups before the hydroxylamine reaction, a free sulfhydryl group was now demonstrable on the papers containing S-acetyl glutathione. When the nitroprusside reagent was added to the mixture of S-acetyl glutathione and BSP after treatment with hydroxylamine, a purple color due to the BSP appeared first and then rapidly faded. A red color attributed to the presence of a free sulfhydryl group was then clearly demonstrable before it also faded. No red color was discernible in the BSP A spot. A thioester linkage in BSP A thus seemed unlikely. It can be concluded, therefore, that no opening of the lactone ring accompanies conjugation of BSP with amino acids.

TABLE V

Reactions for presence of a thioester bond

Compound	Formation of hydroxamic acid	Free sulfhydryl group	
		Before hydroxylamine	After hydroxylamine
50 μl 100 mg % BSP	+	0	0
50 μl 50 mg % S-acetyl glutathione	+	0	+
50 μl 100 mg % BSP + 50 μl 50 mg % S-acetyl glutathione	+	0	+
BSP A 550 mg %			
20 μl	+	0	0
100 μl	+	0	0

d) Thioether linkage with BSP. No evidence was detected of a free sulfhydryl group in unhydrolyzed BSP A. However, after alkaline hydrolysis of the conjugate, approximately one sulfhydryl group was titrated iodometrically for each mole of BSP in two separate pools of BSP A (Table VI). Free BSP did not react with iodine.

These findings, which indicate the presence of bound sulfur in the major BSP conjugates, were supported by results obtained with the qualitative sodium azide-iodine reaction (9). A positive reaction is indicated by the evolution of bubbles of nitrogen. When cysteine was mixed with sodium azide-iodine, bubbles were detected as indicated in Table VII. BSP A also gave a positive reaction. However, when compared with cysteine, the onset of visible reaction was delayed and the bubbles evolved were very fine. Cystine, a compound with bound sulfur, reacted even more slowly than did BSP A. Free BSP did not give a positive reaction.

TABLE VI

Titration of BSP compounds with iodine and thiosulfate solutions

Compound	Molarity of sample	Volume of sample analyzed	0.0005 N iodine consumption	
			Found	Expected
		ml	*ml*	
Free BSP	6.0×10^{-4}	1.0	0*	0
BSP A, Pool 1	6.4×10^{-4}	1.0	1.06*	1.28
BSP A, Pool 2	6.6×10^{-4}	1.0	1.10*	1.32
Mercaptoethanol	5.0×10^{-4}	1.0	1.08†	1.00

* Corresponds to milliliters iodine consumed after alkaline hydrolysis minus milliliters consumed by blank.

† Milliliters iodine used in titration of unhydrolyzed sample.

3. Amino acids detected after hydrolysis of BSP compounds

Glycine, glutamic acid and alanine were identified in hydrolysates of BSP A, BSP B and BSP C. No amino acids were associated with BSP D, the compound which corresponds to free BSP. The ninhydrin spots conforming to glycine and glutamic acid were usually of equal size and color intensity. Occasionally, in hydrolysates of BSP B

TABLE VII

*Results of the sodium azide-iodine reaction**

Molarity of solution	Time in seconds at which bubbles first developed			
	Cysteine (R-SH)	BSP A (R-S-R)	Cystine (R-S-S-R)	BSP
0.0004	12	50–60	90	None
0.0003	30	90	>120	None

* One drop of the test solution was mixed with 1 drop of sodium azide-iodine reagent. There were approximately 32 drops per ml.

and BSP C, the glycine spot was more prominent than the glutamic acid spot. Alanine was always less prominent than glycine and glutamic acid. On some chromatograms, alanine was not detected until the chromatogram was "loaded" with the hydrolysate of the BSP compound.

Oxidation of each hydrolysate was also carried out with hydrogen peroxide and ammonium molybdate on a second chromatogram, as outlined by Dent (10), to facilitate the identification of cysteine. Cysteine and glutamic acid migrate very close to each other and usually are superimposed in the solvent systems used. However, after oxidation, cysteine is converted to cysteic acid, a compound which is easily separated chromatographically from glutamic acid, and thus can be definitely identified. Despite use of this method, cysteine was not detected, even when 30 μg of BSP was placed on the chromatograms. Assuming that BSP is conjugated with one molecule of glutathione, approximately 10 to 12 μg of hydrolyzed glutathione was put on paper. By contrast, cysteine (as cysteic acid) was regularly identified in hydrolysates of glutathione alone and in mixtures of equimolar concentrations of glutathione and BSP when as little as 1.84 μg of hydrolyzed glutathione was put on paper. The cysteic acid spot observed with ninhydrin was less intense than were the glycine and glutamic acid spots.

Since alanine but not cysteine was identified in hydrolysates of BSP conjugates, the possibility that the sulfur of cysteine remained attached to the BSP molecule after HCl hydrolysis was examined. BSP A isolated from a rat given S^{35}-labeled L-cystine was hydrolyzed in 5.9 N HCl at 15 pounds pressure for 3 hours. A chromatogram of the hydrolyzed material was developed, and BSP bands were identified. Radioactivity on

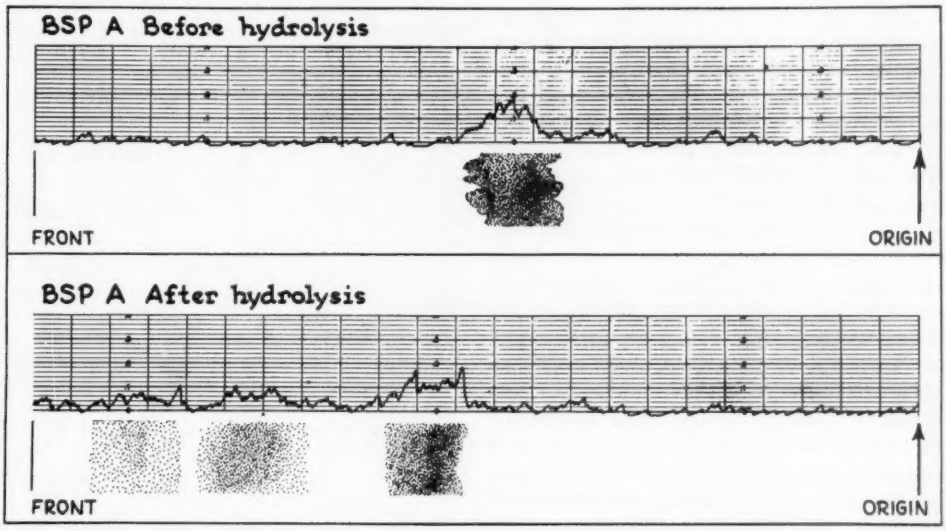

FIG. 4. RELATIONSHIP OF RADIOACTIVITY TO BSP BANDS BEFORE AND AFTER ACID HYDROLYSIS OF BSP A. BSP A was isolated from the bile of a rat that had received S^{35}-labeled cystine. BSP A was partially hydrolyzed in 5.9 N HCl by heating at 100° C for 3 hours at 15 pounds pressure. Drawings of chromatograms of BSP A before and after hydrolysis are shown. Radioactivity was associated with the hydrolytic BSP products.

the chromatograms was then located by a chromatogram scanner.[3] Three BSP-containing bands with $R_{f's}$ of 0.56, 0.77 and 0.87, respectively, were seen after hydrolysis. The R_f of unhydrolyzed BSP A was 0.48 and that of free BSP, 0.76. When free BSP is subjected to acid hydrolysis and then chromatographed, at least two compounds are identified. One travels with the same R_f as does free BSP, the other moves more quickly (*2*). Therefore, the BSP bands moving with $R_{f's}$ of 0.77 and 0.87 in the hydrolyzed sample of BSP A no doubt corresponded to these BSP moieties. Radioactivity was associated with all three BSP compounds (Figure 4), indicating that the S^{35} of cysteine remains attached to the BSP molecule after hydrolysis.

DISCUSSION

The present studies indicate that cysteine as well as glycine and glutamic acid is present in the BSP conjugates. Thus, radioactivity conforming in shape and position with the BSP conjugates, but not free BSP, was regularly identified on chromatograms of bile collected from animals that had received S^{35}-labeled reduced glutathione and S^{35}-labeled L-cystine. The radioactivity was intimately associated with the BSP compound since it could not be removed by passing BSP A through a Dowex-50× 200 to 400 mesh ion exchange column. Incorporation of the radioactive sulfur into BSP conjugates need not be attributed to cysteine conjugation, however. Radioactivity might appear in the BSP compounds by S^{35} exchange with the sulfonic acid groups or by formation of an ethereal sulfate with the hydroxyl groups of BSP. Both of these possibilities seem to have been excluded. Since radioactivity appeared only in the BSP conjugates when S^{35}-labeled glutathione and S^{35}-labeled L-cystine were administered, sulfur exchange would have occurred in the conjugates and not in free BSP. It was reasoned, therefore, that sulfur exchange should be indicated by a lowering of the specific activity of the BSP conjugates and not of free BSP when S^{35}-labeled BSP was administered. The specific activities of BSP A and BSP D (free BSP) eluted from paper chromatograms did not differ significantly. In addition, no radioactivity appeared in the BSP conjugates in the rats given inorganic $S^{35}O_4$. Sulfur exchange was therefore ruled out. Formation of an ethereal sulfate of BSP appears to be excluded by the observation that the ratio of S^{35} counts per minute per unit optical density of BSP in BSP A was not altered under conditions in which hydrolytic cleavage of an ethereal sulfate would be expected. This finding was supported by the less specific inorganic $S^{35}O_4$ experiments, in which no evidence was found of incorporation of radioactivity into the BSP conjugates.

It seemed likely, therefore, that the association of radioactivity with the BSP conjugates in animals receiving S^{35}-labeled glutathione and L-cystine was due to incorporation of cysteine in the BSP conjugates. Chromatograms of BSP conjugates hydrolyzed with 5.9 N HCl failed to reveal this amino acid, however. In part, this could be explained by destruction of cysteine which is known to occur during hydrolysis with strong HCl (14). Nevertheless, such an explanation could not account for the inability to find cysteine when hydrolysates containing up to 30 μg of BSP were placed on the chromatograms. Such chromatograms would contain approximately 4.3 μg of cysteine, assuming that one mole of cysteine is conjugated with one mole of BSP. Even the much smaller quantity of cysteine contained in 1.8 μg of reduced glutathione, approximately 0.7 μg, was readily detected chromatographically when glutathione either alone or containing an equimolar amount of free BSP was hydrolyzed with 5.9 N HCl. The presence of alanine on the amino acid chromatograms suggested another possibility, namely, that the sulfhydryl group of cysteine was split off during acid hydrolysis. Indeed, this was confirmed by finding radioactivity associated with the hydrolytic products liberated during hydrolysis of BSP A obtained from a rat that had received S^{35}-labeled L-cystine. From the size and color intesity of the alanine spot on amino acid chromatograms, desulfuration of cysteine with liberation of alanine seemed to be more significant quantitatively than was destruction of cysteine to non-ninhydrin-reacting products in explaining the inability to detect cysteine chromatographically.

In the present studies, evidence of a thioether bond between the sulfhydryl group of cysteine and BSP was adduced from the observation that ap-

[3] Model RSC-5A chromatogram scanner manufactured by Atomic Accessories, Inc.

proximately one sulfhydryl group was titrated for one mole of BSP after alkaline hydrolysis of BSP A, the major conjugate in the rat, whereas no titrable sulfhydryl group was present prior to hydrolysis. Similar results would have been obtained if the sulfhydryl group of cysteine was conjugated with the lactone ring of BSP in thioester linkage. The absence of a free sulfhydryl group after reaction of BSP A with hydroxylamine excluded a thioester bond, however. Absence of a free sulfhydryl group in the intact BSP conjugate also implies that cysteine is not bound in peptide linkage. The results obtained in the reaction between the BSP conjugates and hydroxylamine afford additional evidence for this. Free BSP contains a lactone ring which is capable of forming a hydroxamic acid. If the lactone ring were engaged in peptide bond formation, hydroxamic acid formation would be unlikely. The observation that the conjugates BSP A and BSP C, like free BSP, formed hydroxamic acids ruled out such a linkage. Since cysteine can be easily identified in hydrolysates of glutathione, a compound in which this amino acid exists in peptide linkage, it seems likely that difficulty in identifying cysteine in hydrolysates of BSP conjugates is related to the existence of the thioether bond. During hydrolysis of the BSP thioether linkage with strong HCl, the sulfhydryl group tends to remain attached to the BSP molecule.

In confirmation of earlier studies (2), glycine and glutamic acid were identified in hydrolysates of BSP conjugates. It was previously pointed out that these amino acids are found occasionally when control bile is chromatographed and the areas with the same $R_{f's}$ as the BSP compounds are eluted, hydrolyzed and then chromatographed for amino acids. The compounds in control bile that yield these amino acids on acid hydrolysis can be removed, however, by passing them through a Dowex-50× ion exchange column (2). BSP conjugates are not altered by this procedure. Therefore, eluates containing BSP compounds are regularly passed through such a column before hydrolysis and amino acid chromatography in this laboratory. Glycine and glutamic acid were regularly identified in all of the BSP conjugates. The amounts of glycine and glutamic acid liberated by hydrolysis were not quantitated chemically. However, approximately one mole of glycine and of glutamic acid were usually present for each mole of BSP, as estimated by paper chromatography. Alanine, which we consider to be cysteine minus its SH group, was also present in chromatograms of the BSP conjugates. Thus glycine, glutamic acid and cysteine were bound to BSP to form the BSP conjugates appearing in rat bile. These three amino acids compose the tripeptide glutathione. It may be concluded, therefore, that BSP is conjugated in the liver of the rat with glutathione, conjugation occurring through the sulfhydryl group of cysteine. We would also interpret our earlier finding of the presence of glycine, glutamic acid and alanine in hydrolysates of human BSP conjugates (2) as indicating that BSP is conjugated with glutathione in man. These conclusions are in essential agreement with the findings of Javitt, Grodsky and their respective associates. Thus, Javitt, Wheeler, Baker and Ramos, in a preliminary report, suggested that BSP is excreted as a glutathione conjugate in the dog (5). Grodsky, Carbone and Fanska "concluded that BSP is excreted at least in part as a mercaptide with cysteine or the peptide glutathione," in man (4). These latter authors are not certain about the presence of glutathione, however, since they feel that glycine and glutamic acid are possible contaminants of the BSP metabolites.

At least four distinct BSP bands can be separated by paper chromatography of rat bile (2). One band, BSP D, corresponds to free BSP. The other three, BSP A, BSP B and BSP C, are amino acid conjugates, with BSP A the major one. Our findings indicate that each of these latter bands contains BSP and glutathione. This implies that they are isomers with mobility in the solvent system differing with site of conjugation. We have no direct evidence for or against conjugation of BSP with 2 moles of glutathione, although visual analysis of the quantity of amino acids on paper chromatograms suggests that only 1 mole of glycine is present for each mole of BSP. The observation that BSP B and BSP C may yield more glycine than glutamic acid on hydrolysis suggests that these BSP bands may also contain breakdown products of BSP-glutathione such as BSP-cysteinyl glycine and BSP-cysteine. Quantitatively, however, BSP-glutathione is the major conjugate found in rat bile.

Our demonstration that glutathione is conju-

gated in thioether linkage with BSP may be considered as additional evidence for the view that glutathione is involved in the formation of mercapturic acids (15). The glutathione thioether would then be split to a cysteine thioether by the action of glutathionase (16). In this regard, it is of interest that BSP has been found to be a potent inhibitor of glutathionase (17), an observation which is consistent with our finding that BSP-glutathione is the major conjugate in rat bile.

The present studies also provide additional information about the structure of the BSP molecule appearing in the BSP conjugates. The sulfonic acid groups do not appear to be removed or engaged in sulfur exchange. The hydroxyl groups, at least in the major BSP compound, BSP A, are not conjugated with sulfate and earlier studies (1, 2) excluded glucuronide formation. The lactone ring remains intact. Sites available for glutathione conjugation include, therefore, the positions occupied by the four bromine atoms, and the free positions on the two phenolic rings. Krebs and Brauer found that the ratio of bromine to sulfur in Compound III of the rat (probably identical with BSP A) was the same as that in free BSP (1), suggesting that glutathione is conjugated at one of the free positions on the phenolic rings in BSP A. The determination of bromine to sulfur ratios will be of importance in elucidating the site of conjugation in the other BSP compounds.

SUMMARY

The three ninhydrin-positive sulfobromophthalein sodium (BSP) compounds appearing in rat bile have been shown to consist of BSP conjugated with the amino acids cysteine, glycine and glutamic acid. These amino acids are the constituents of glutathione. Therefore, it is concluded that most of the BSP excreted in rat bile is BSP-glutathione. BSP A, the major BSP compound in rat bile, appears to be BSP-glutathione. BSP B and BSP C may consist of isomers of BSP-glutathione plus breakdown products of this compound such as BSP-cysteinyl glycine and BSP-cysteine. Conjugation was shown to occur through the sulfhydryl group of cysteine with the formation of a thioether linkage.

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EFFECTS OF HUMAN GROWTH HORMONE IN MAN *

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Beck, McGarry, Dyrenfurth and Venning (1) first demonstrated activity of purified monkey and human pituitary growth extracts in man and this observation has been confirmed by Ikkos, Luft and Gemzell (2), Raben (3), Pearson, Lipsett, Greenberg and Ray (4), Henneman and Bergenstal and their co-workers (5, 6) and others (7). Studies in man have employed pituitary growth extracts prepared by the methods of Raben (8), Wilhelmi, Fishman and Russell (9) and Li and Papkoff (10). This paper summarizes balance and clinical data from 10 patients treated for 1 day to 24 months with the Raben preparation of human growth hormone (HGH).

METHODS

Pituitary glands were collected at autopsy,[1] stored in cold acetone, and extracted by Dr. Maurice Raben (8).[2] The dry powder appeared to be stable at room temperature. It was dissolved in 1.0 ml 0.1 N HCl per 100 mg dry powder (final pH of solution, about 3.5). The solution was diluted with distilled water. Such hydrochloric acid solutions of HGH maintained their potency after refrigerator storage for at least 6 months. Intramuscular injections of these extracts were painful. Dilution with equal parts of 1 to 2 per cent procaine-HCl did not decrease effectiveness but rendered the injection painless; such procaine-growth extract preparations precipitated and had to be resuspended by shaking before use. Four lots of growth hormone were used in these studies but no differences between lots were noted.

Ten patients were studied under complete balance conditions. General procedures were as previously described (11, 12) except that free access to water was permitted and water intake was measured. Clinical data regarding the patients are presented in Table I. Complete balance data on 7 patients are presented in Figures 1 through 7; data from 2 other patients and the details of change in blood levels of carbohydrate and fat intermediary metabolites are reported elsewhere (13). Balance data on M.A. during HGH administration confirmed the other studies; they are not presented because bizarre postcontrol data[3] suggested a systematic error limited to this phase of the study.

The ratios (11) of nitrogen to phosphorus, sodium and potassium in normal muscle and of calcium to phosphorus in normal bone were used to calculate the theoretical balances of the HGH-treated patients.

RESULTS

Linear growth. Table II shows that the growth rate was definitely stimulated in three patients on prolonged HGH therapy and was probably stimulated in two other patients treated for only one month. The fall in growth rate of S.M., D.Z. and J.C. on stopping HGH adds further validity to the conclusion that HGH stimulated growth. The growth rate of D.Z. and J.C. has tended to

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[1] Appreciation is expressed for the cooperation of the Pathology Departments of the following hospitals: Massachusetts General, Beth Israel, Peter Bent Brigham, Deaconess, and Boston City in Boston; the Newton-Wellesley in Newton, Mass.; the Rhode Island General and Pawtucket Memorial in Rhode Island; St. Luke's, Mt. Sinai, Presbyterian, Montefiore, Albert Einstein, Roosevelt and Kings County in New York; and Presbyterian, University of Pennsylvania, Temple University and Jefferson Medical School in Philadelphia. The efforts of Drs. Robert L. Neilsen, Stanley Wallach and Roger Melick in collecting human pituitaries made possible the extent of the studies herein reported.

[2] The experiments to be reported owe much to Dr. Raben who prepared the growth extract from human pituitaries and to Drs. Raben, J. C. Beck and O. Pearson who shared their experiences with common lots of Raben extract prior to public presentation of results.

[3] During 18 days postcontrol urinary nitrogen, phosphorus, sodium and potassium failed to return to forecontrol levels despite lack of other evidence for continuing HGH effect, for spontaneous resumption of normal growth, for weight change or for undetected change in intake.

TABLE I

Clinical data on patients

Patient	Chronological age	"Bone age"	Height	Weight	Thyroid status	Adrenal status: Urine 17-OHCS*	Adrenal status: Water test†	Other treatment	Diagnosis
	yrs	*yrs*	*in.*	*lbs*					
S.M.	14	8.75	50	53	See across	1.6		Triiodothyronine	Craniopharyngioma, hypopituitarism
M.A.	13	12	55	78	Normal	0.4	pos.		Craniopharyngioma, hypopituitarism
M.C.	27	15.5	67	109	Normal	1.1	pos.	Triiodothyronine	Idiopathic hypopituitarism (since childhood)
B.D.	49	Adult	68	440	Normal	4.5			Obesity
D.Z.	9	6	39	31	Normal	1.0			Short stature, delayed bone age (? hypopituitarism)
F.D.	62	Adult	69	130	(Cachectic)	(Cachectic)			Malignant melanoma
S.G.	21	Adult	57	94	Normal	1.7 17-KS = 3.0			Short stature, gonadal dysgenesis
J.C.‡	15	6	50	68	Normal	0.6			Short stature, delayed bone age (? hypopituitarism)
E.S.	44	Adult	61	124	Normal	0			Addison's disease
L.C.‡	66	Adult	63	113	Normal	0.5			Idiopathic hypopituitarism (onset age 60)

* Porter-Silber chromagens, mg per 24 hours (14); normal range for adults, 2.0 to 7.5 mg per 24 hours.
† Reference 15.
‡ Balance data reported elsewhere (13).

decrease with time on treatment over 21 to 24 months.

Nitrogen metabolism. Nitrogen retention was produced in all patients. Nitrogen retention was slight the first day of treatment with 0.2 to 10 mg HGH per day; it was maximal (4.5 to 8.0 g per day) on the third to sixth days of therapy; usually began to wane after 6 to 9 days and retention of 40 to 60 g of nitrogen; and after 30 days' treatment it was almost undetectable. After 12 days off HGH, Patient B.D. once more responded to HGH administration with marked nitrogen retention. In general there was slightly greater nitrogen excretion after growth hormone was discontinued than during fore-control, although this "rebound" loss did not match the retention in degree or extent. Nitrogen retention was least marked in S.G. (short stature, gonadal dysgenesis), F.D. (malignant melanoma with cachexia) and E.S. (Addison's disease). No nitrogen retention was produced by 0.1 mg HGH in S.M.; some retention was produced by 0.2 mg HGH daily in D. Z., marked retention was produced by 2.5 to 5.0 mg in S.M., M.A., M.C., B.D. and D.Z.; in S.G. and F.D. 10 mg HGH daily appeared to be more effective than were 2.5 and 5.0 mg, respectively. Nitrogen absorption was unaltered by HGH. Fasting blood urea nitrogen decreased 30 to 40 per cent during HGH administration in four patients, M.A., M.C., B.D. and D.Z. (all of whom showed marked nitrogen retention), and failed to change significantly in Patients S.G. and E.S. (who showed little nitrogen retention). Fasting serum α-amino nitrogen was unaltered as compared with postcontrol values in S.M. and F.D. There was a suggestive decrease in urinary total creatinine during HGH in S.M.

Phosphorus metabolism. Phosphorus retention on HGH was due primarily to decrease in urinary phosphorus and paralleled nitrogen retention. Absorption of phosphorus was slightly improved on HGH. Total serum inorganic phosphorus

TABLE II

*Linear growth rate on human growth hormone**

Hypopituitary patient	Average normal growth rate for "bone age"†	Rate of growth before HGH	Dose	HGH therapy schedule	Duration	Rate of growth on HGH	Rate of change of bone age‡		Rate of growth off treatment
							Before treatment	On treatment	
	cm/mo	*cm/mo*	*mg*		*mos*	*cm/mo*	*mos/mo*		*cm/mo*
S.M.	0.46	0.21	2.0	q.o.d.	9	0.35	0		0.05§
M.A.	0.46	0.21	2.5	q.d.	1	0.50			0.13§
M.C.	0.50	0.15	2.5	q.d.	1				
D.Z.	0.48	0.25	2.0	q.d. 2 wks on 2 wks off	5	1.00	0.5	} 1	
			2.0	q.d.	4	0.48			
			2.0	q.d. 2 wks on 2 wks off	5	0.12			
			4.0	q.d. 2 wks on 2 wks off	4	0.62			
			0.0		3	0.0			0.0
J.C.	0.48	0.27	2.0	q.o.d.	7	0.39§	0.2	} 1	
			2.0	q.d. 2 wks on 2 wks off	5	0.42§			
			0.0		5	0.10§			0.10§
			4.0	q.d. 2 wks on 2 wks off	7	0.29§			

* Growth rate before therapy was calculated over approximately two-year periods. The height measurements represent numerous determinations consistent with each value.

† Reference 16.

‡ Bone age evaluation depends upon size as well as shape of bones. HGH increases size of bones; these data are not adequate to predict whether HGH will accelerate the maturation and closure of epiphyses.

§ Patients received cortisone, 10 to 25 mg daily, during these periods of observation. Such therapy by itself may further slow the slow rate of growth observed in most children with hypopituitarism (17).

rose significantly in five patients and did not change in two patients (F.D. and S.G.) who similarly showed little nitrogen response.

Calcium metabolism. The changes in calcium metabolism were unique. Calcium retention (0 to 400 mg per day) occurred in three patients (B.D., F.D. and D.Z.) during therapy; more marked and more persistent calcium retention occurred after stopping HGH in four patients (S.M., M.A., S.G. and D.Z.). Indeed, the calcium retention which followed HGH did not wane within the 30, 18, 4, and 12 days of observation off HGH in Patients S.M., M.A., S.G. and D.Z., respectively. Fecal calcium decreased on HGH to approximately the same extent that urinary calcium increased in six of seven patients so studied. The further retention of calcium following HGH was due to a decrease in urinary as well as in fecal calcium. It may be significant that increase in urinary calcium occurred in six of seven patients, that B.D. and F.D., with mature skeletons, showed calcium retention during HGH while S.M., M.A. and D.Z., with open epiphyses, showed calcium retention more marked after HGH was stopped.

Serum calcium rose slightly in one patient (S.M.) and was unchanged in another (M.C.).

Serum alkaline phosphatase. Serum alkaline phosphatase showed no consistent response during 6 to 36 days of HGH treatment.

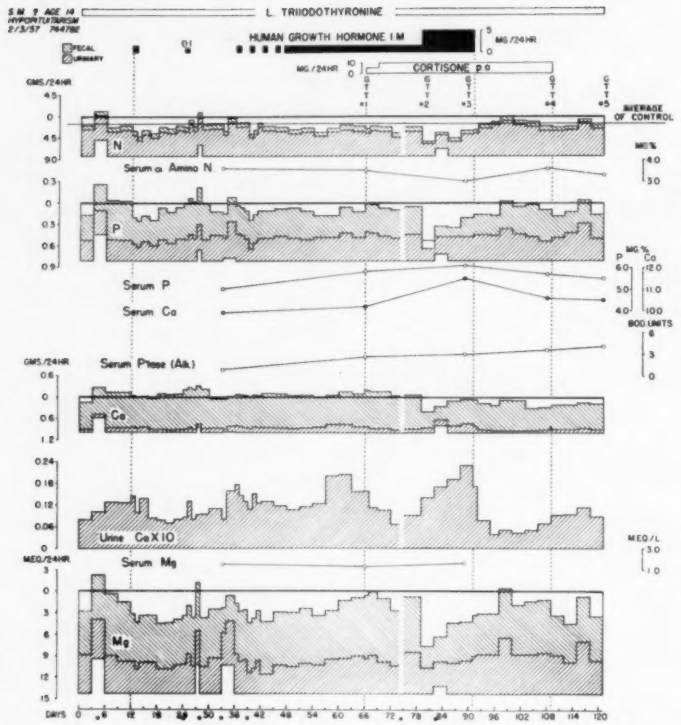

FIG. 1A. EFFECT OF HGH IN S.M., A 14 YEAR OLD GIRL WITH HYPOPITUITARISM. Presented are the balance data for nitrogen, phosphorus, calcium and magnesium. The scales are in the same proportion as the proportions in which these constituents are found in most tissues (11). There is a heavy zero line for each substance. The intake is measured down from the zero line; the urinary and then the fecal excretion are charted up from the intake line. Negative balance appears as a hatched area above the zero baseline; positive balance is represented by a clear area below the zero baseline. An ancillary baseline representing the average balance of the fore-control has been superimposed on the nitrogen balance data to facilitate comparison with the treatment and postcontrol periods. HGH was administered as follows: a single intramuscular injection of 1.0 mg on Day 13; after 12 days a single injection of 0.1 mg; after 12 more days injections of 1.0 mg every 3 days; and finally, daily injections of 1.0 mg and then of 5 mg. Lot 2 was used throughout the study. The results of the five glucose tolerance tests (G.T.T.) are presented elsewhere (13). The glucose for these tests was withdrawn from the regular glucose ration for that day so that the total caloric intake remained constant. Asterisks under the "days" scale indicate vomitus or refusals, the values for analysis of which were substracted from intake.

S.M.'s growth rate became subnormal at 3 years of age. She underwent partial removal of a craniopharyngioma and pituitary at 7.5 years. At age 11, studies revealed mild diabetes insipidus, a "bone age" of 8.75 years, urinary 17-ketosteroids = 0.3 mg per 24 hours, urinary hydroxycorticosteroids (Porter-Silber chromagens) = 1.6 mg per 24 hours, and a negative test for urinary gonadotropins at 6.5 or more mouse units per 24 hours. Thyroid replacement therapy was changed to L-triiodothyronine, 75 μg daily, one month prior to this study.

S.M. received 2 mg HGH (plus thyroid 120 mg and cortisone 25 mg daily and Pitressin Tannate in Oil, 0.5 ml every 3 days) every other day from September, 1957 to April, 1958 when HGH was discontinued due to the development of nausea, vomiting, headache, appearance of bitemporal hemianopsia and roentgen evidence of further destruction of the sella turcica. Following discontinuation of HGH these symptoms gradually disappeared and visual fields improved. As of March, 1960 there has been no evidence of further growth of the craniopharyngioma.

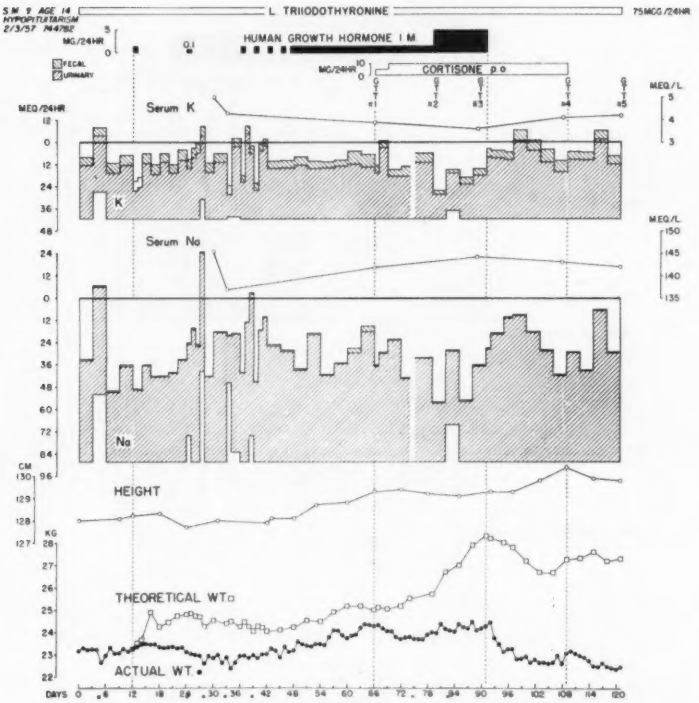

FIG. 1B. FURTHER EFFECTS OF HGH IN S.M. The theoretical weights in this and subsequent studies are based on the caloric discrepancy of the fore-control plus the weight equivalent (11) of changes in nitrogen, potassium and sodium metabolism on HGH.

Potassium metabolism. HGH induced potassium retention in 10 subjects. The reduction in urinary potassium was usually maximal the first day[4] of treatment. Potassium retention was slightly greater than that predicted from the nitrogen balance if the K : N ratio = 2.5 of normal muscle (11) is used. Potassium retention was particularly marked the first day on HGH and it waned on prolonged therapy, as did the nitrogen balance. Fecal potassium was also somewhat less during HGH. Urinary potassium was greater than predicted from the nitrogen excretion the first few days off HGH. This "rebound" was of less magnitude than the total retention during HGH, but was similar in magnitude to the retention the first two days on HGH.

Sodium metabolism. Sodium retention regularly occurred on HGH; it was apparent the first day but usually was not maximal until the second or third day of treatment. More milliequivalents of sodium than of potassium were retained. Sodium retention waned on prolonged therapy. On stopping HGH, sodium excretion was increased and the negative sodium balance was equal to or less than the total sodium retained during treatment. HGH decreased urinary sodium excretion in a patient with adrenal insufficiency (E.S.).

Magnesium metabolism. Magnesium retention occurred in four patients on HGH; magnesium balance was unaltered in three. Retention in

[4] However, hourly urinary potassium was not altered during the first eight hours after an injection of HGH in one subject.

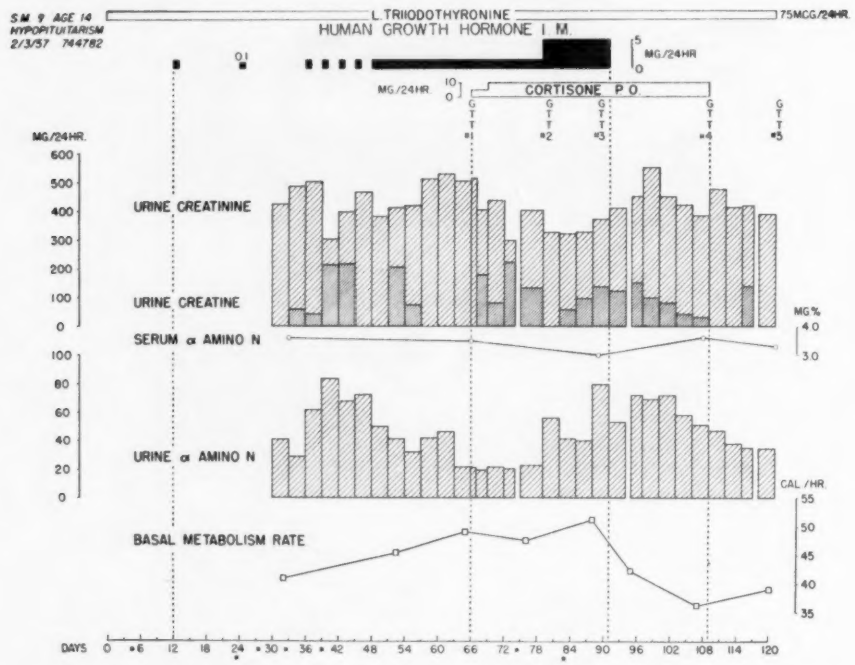

FIG. 1C. FURTHER EFFECTS OF HGH IN S.M. Variations in serum α-amino nitrogen and urinary creatine and α-amino nitrogen did not appear related to HGH administration in S.M. (and in M.A.). There may have been a decrease in urinary total creatinine in S.M., particularly on the larger doses of HGH.

each patient was due to a decrease in fecal excretion. Serum magnesium was constant.

Body weight. Weight increased in all patients except those (B.D., F.D. and S.G.) who were on calorically-inadequate diets.

In every experiment the actual weight during HGH therapy was less than the theoretical weight (11) (based on the caloric discrepancy [5] of the fore-control period plus changes in nitrogen, potassium and sodium balance). A discrepancy between theoretical and actual weight appeared and increased rapidly during the initial phases of HGH administration and did not continue to change during postcontrol. If this discrepancy were due to a systematic error or to incorrect factors in calculation, one would expect continued deviation of actual and theoretical weights throughout the postcontrol period.

Basal metabolic rate. The basal metabolic rate,

[5] The caloric discrepancy represents the daily discrepancy between caloric intake and caloric expenditure. It is calculated on the assumptions that caloric expenditure is constant each day, that the degree of hydration is the same at the beginning and end of calculations, and that fat gain or loss accounts for all weight gain not accounted for by the balances of nitrogen, sodium and potassium.

TABLE III

Cumulative discrepancy between actual weight and theoretical weight on last day of HGH administration

Patient	Theoretical weight − actual weight	Duration of treatment
	kg	*days*
S.M.	4.5	78
M.A.	6.0	30
M.C.	0.5	30
B.D.	3.0	42
S.G.	2.0	20
D.Z.	1.8	12

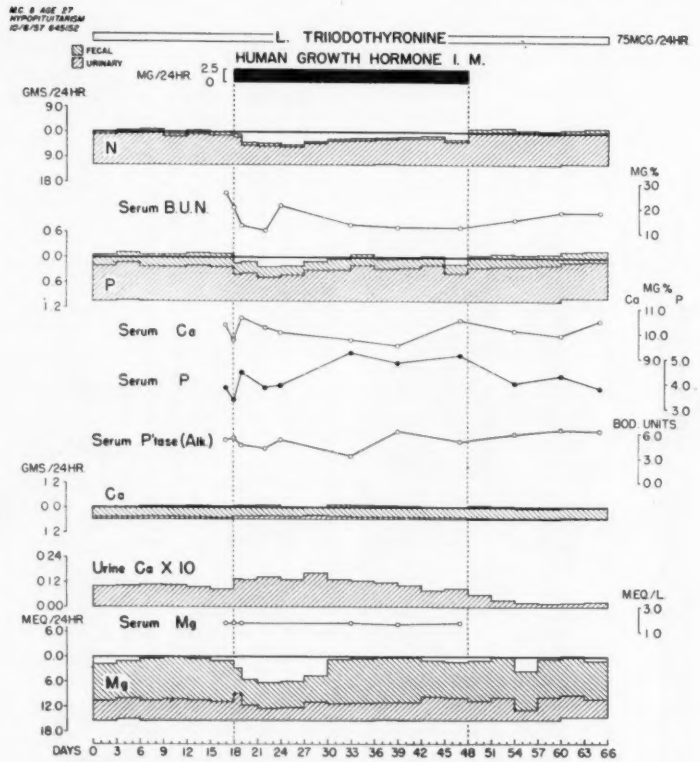

FIG. 2A. EFFECT OF HGH IN M.C., A 27 YEAR OLD MAN WITH HYPOPITUITARISM. When M.C. was first seen at age 20 for headaches and emotional instability he was dwarfed (height 57″), devoid of body hair and eunuchoid. "Bone age" was 10 years, urinary FSH assay was negative at the lowest levels tested, and urinary 17-ketosteroids were 0.4 mg per 24 hours. Immediately prior to this study BMR was −37, PBI 4.18 μg per 100 ml, and RaI uptake 38.8 per cent. There was no evidence of sexual maturation, bone age was 15 years, water test was positive, and urinary 17-hydroxycorticoids were 1.1 mg per 24 hours. L-triodothyronine, 75 μg per day, was administered throughout the study to ensure normal thyroid hormone status. Lot 3 HGH was used throughout the study.

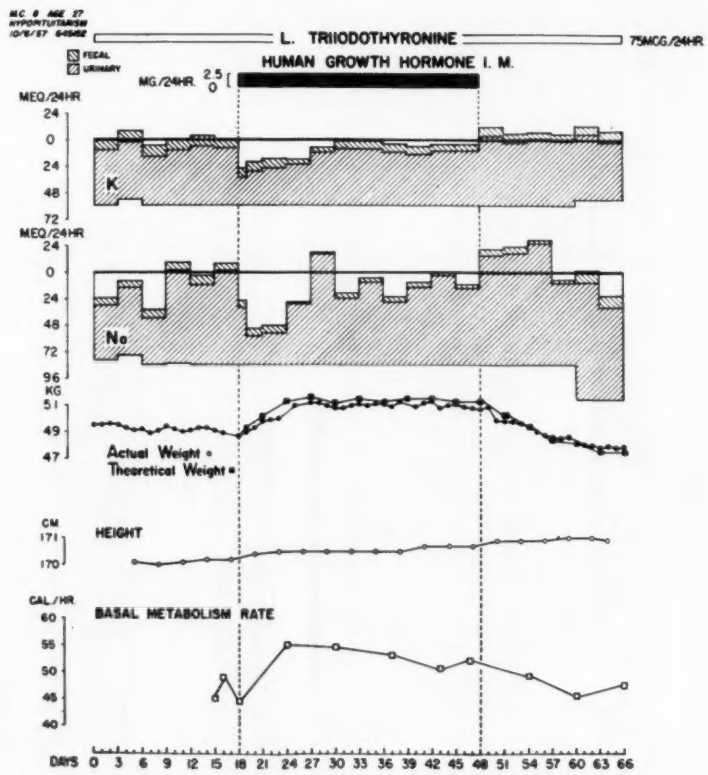

FIG. 2B. SODIUM AND POTASSIUM BALANCE, WEIGHT, HEIGHT AND BASAL METABOLIC RATE DURING HGH ADMINISTRATION TO M.C.

expressed as calories per hours, rose 20 to 25 per cent in the three patients (S.M., M.A. and M.C.) so studied.

Appetite. Beck and associates (1) reported marked anorexia in the first patient treated with large doses of HGH. Mild loss of appetite was reported on direct questioning of each of our subjects. Since loss of appetite is the rule on prolonged constant diets, it is probably more significant that our patients volunteered that they noted improvement in appetite when HGH was discontinued. The two patients on prolonged treatment noted mild anorexia on HGH as compared with appetite during two-week periods off HGH.

Hormone assays. Urinary 17-ketosteroid and 17-hydroxycorticosteroids, serum protein-bound

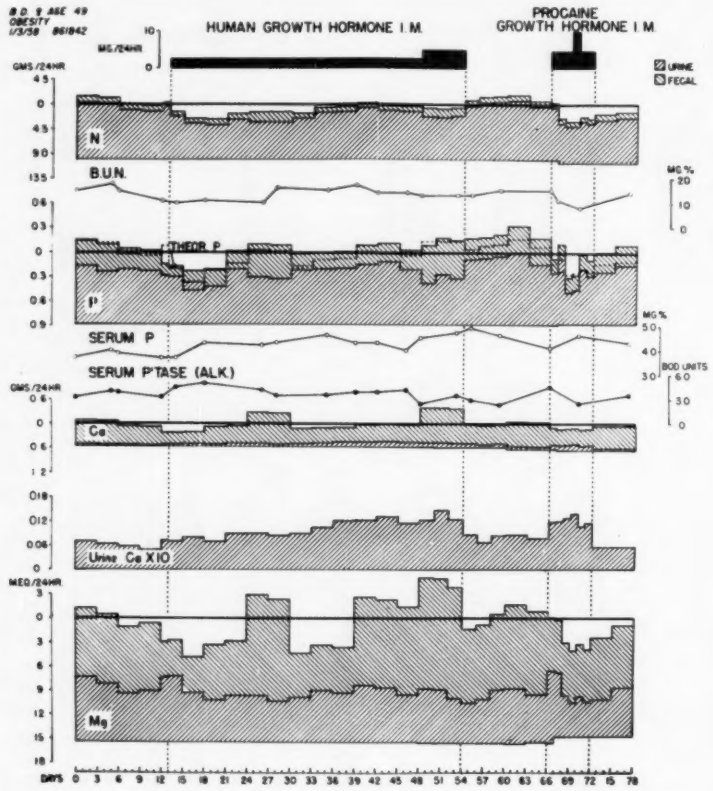

FIG. 3A. THE EFFECTS OF HGH IN B.D., A 49 YEAR OLD WOMAN WITH MARKED OBESITY. Despite a preceding period on the 800 calorie constant diet the patient was still adapting during the 13 days of fore-control as shown by gradually diminishing negative balances. HGH was given as single daily injections at 6 a.m.; on Day 69 the patient received 5 mg growth hormone at 6 a.m. and at 6 p.m.; on Days 70 and 71 she received 5 mg growth hormone at 6 p.m. The time of administration did not affect the net effects of the hormone. B.D. received Lot 3 HGH Days 12 through 27 and Lot 4 HGH for the remainder of the study. The addition of an equal volume of 2 per cent procaine-HCl to the HGH did not decrease its effectiveness. The dotted line on the phosphorus balance represents the "theoretical" phosphorus balance calculated from the nitrogen and calcium balances.

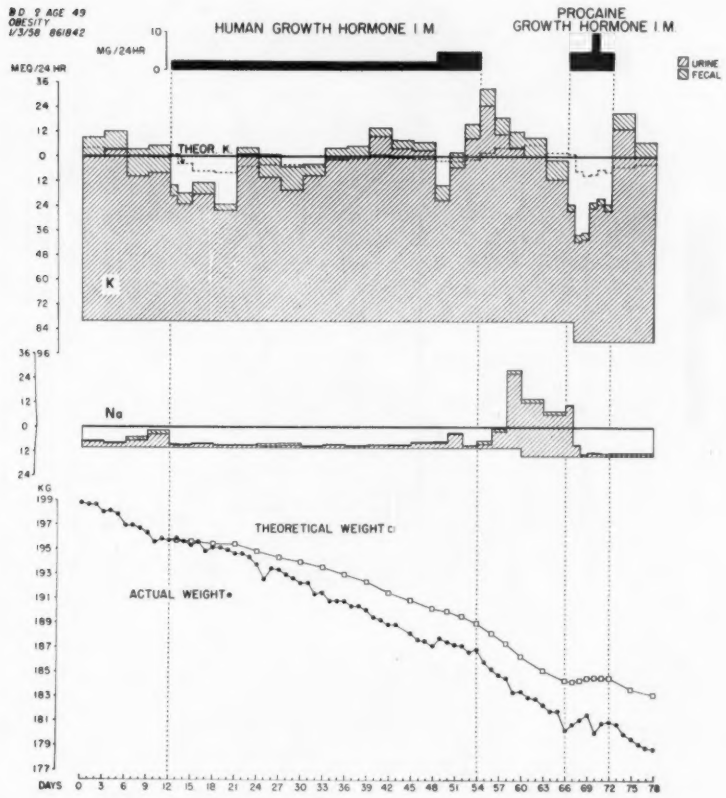

FIG. 3B. POTASSIUM AND SODIUM BALANCES, AND ACTUAL AND THEORETICAL WEIGHTS DURING HGH ADMINISTRATION TO B.D. The dotted line on the K balance represents the theoretical balance predicted from the nitrogen balance. Note in particular the unaccounted-for K retention on starting HGH and the unaccounted-for K loss on stopping HGH. Fasting serum levels of cholesterol, cholesterol esters, total fatty acids and phospholipids were unaltered by HGH in this patient. The actual weight falls below the theoretical weight and this discrepancy appears during HGH therapy and changes hardly at all when HGH is discontinued.

iodine and radioiodine uptake were unaltered by HGH administration in three patients with hypopituitarism. Patient S.M. had mild diabetes insipidus; her constantly dilute urine was not more concentrated during HGH administration.

Reactions to human growth hormone. Patients complained of mild to moderate pain at the site of intramuscular injections of HGH dissolved in dilute HCl. Addition of an equal volume of 1 to 2 per cent procaine-HCl rendered the injections painless. In no patient was a rash or any other sign of allergic reaction noted. Dr. Jacob Lerman (18) found no significant levels of serum precipitins to HGH in two patients receiving prolonged therapy.

Route and time of injections. S.M. received the growth hormone injections subcutaneously as well as intramuscularly without evident alteration in response. The injections of HGH in B.D. were administered at 6 p.m. for three days; the

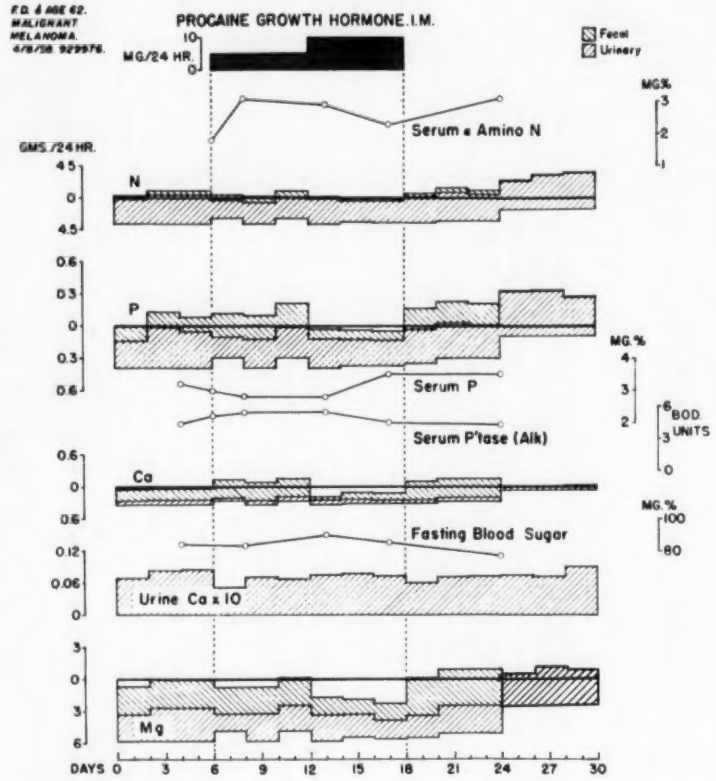

FIG. 4A. THE EFFECT OF HGH IN F.D., A 62 YEAR OLD MAN EMACIATED DUE TO METASTATIC MALIGNANT MELANOMA. The daily caloric intake was 800 (as in the B.D. study) except where decreased by refusals and vomiting. Note the small nitrogen intake. Lot 4 HGH was used throughout this study.

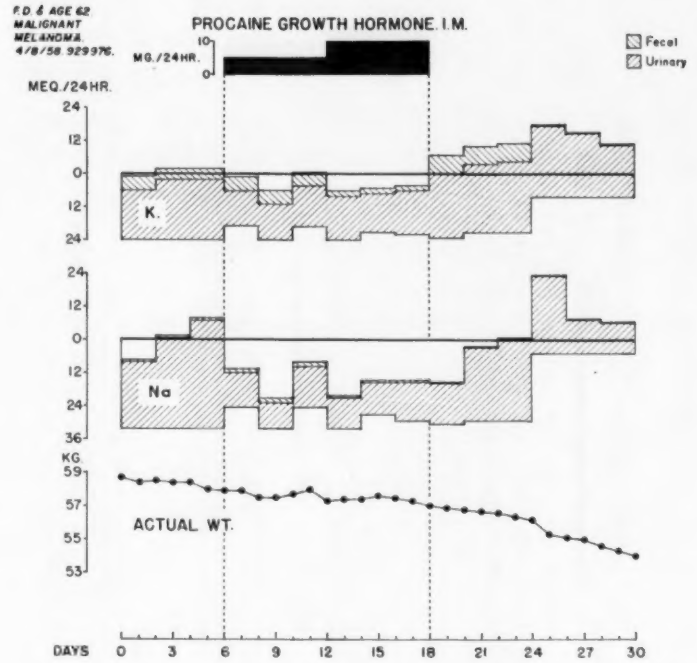

FIG. 4B. POTASSIUM AND SODIUM BALANCES AND BODY WEIGHT DURING HGH ADMINISTRATION TO F.D. Melanoma biopsies before and during growth hormone did not appear histologically dissimilar and roentgenographic examinations of the lungs failed to show evidence of accelerated tumor growth during HGH administration.

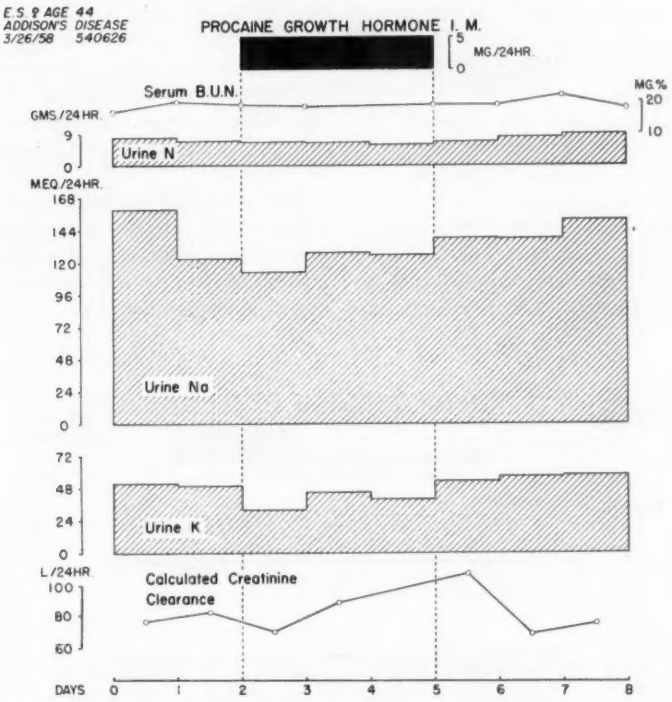

FIG. 5. EFFECT OF HGH IN E.S., A 44 YEAR OLD WOMAN WITH ADDISON'S DISEASE. E.S. received a constant diet throughout the entire study. No fecal measurements were made. Lot 4 HGH was used throughout this study.

response to evening injections was similar to that observed with early morning injections.

DISCUSSION

HGH was effective in ten patients. Linear growth was stimulated in three and probably stimulated in two other of the five patients with open epiphyses. Balance studies revealed retention in all patients of nitrogen, phosphorus, sodium, potassium and calcium in the proportions characteristic of muscle and bone. The most consistent and sensitive indices of response to HGH were the decreases in urinary nitrogen, sodium and potassium. Weight gain was less than that predicted (17) from the nitrogen, sodium and potassium balances. This discrepancy may reflect in part increased fat catabolism since metabolic rate rose and plasma free fatty acids (13) increased. The magnitude of the discrepancy also suggests that the ratio of constituents of normal tissues may not be applicable to hypopituitary patients treated with HGH.

From these studies it would appear that the effective dose of HGH in the human is more than 0.1 mg per day, that the response to 5 to 10 mg per day is greater than it is to 0.2 to 2.5 mg per day, and that 5 to 10 mg daily produces more rapid nitrogen retention, at least initially, than is usually observed (19) in normal children. Beck's original study suggested that 10 to 80 mg per day of human or monkey growth hormone was excessive in that anorexia, oliguria and transient psychic changes resulted. A single injection of HGH may alter nitrogen excretion for up to 10 days; after prolonged administration nitrogen retention ceases promptly on stopping HGH. There

does not appear to be "rebound" excretion of protoplasmic constituents to a degree comparable with the degree of retention during therapy. This suggests that one does not "ungrow" if growth hormone stimulation ceases. By contrast, the nitrogen retained during testosterone therapy is usually lost as quickly as it is retained as soon as therapy ceases.

HGH was about equally effective in producing nitrogen retention in intact as in hypopituitary patients. Smaller responses were observed in F.D., probably due to lack of dietary calories and body fat stores; in E.S., who had Addison's disease; and in S.G., whose short stature is an unexplained part of gonadal dysgenesis and possibly is due to unresponsiveness to growth hormone. These and other possible explanations for differing responsiveness require further studies for confirmation.

The decreasing effect of HGH on prolonged administration in man is recorded. It is likely that HGH initially stimulates rapid retention of nitrogen and other constituents of tissues in a special, perhaps labile, form. Once this require-

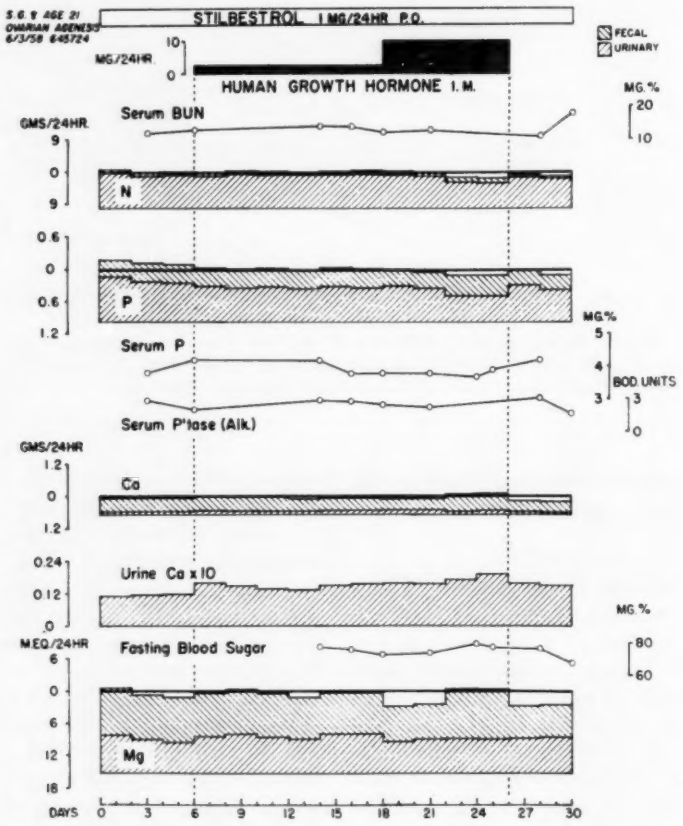

FIG. 6A. EFFECT OF HGH ADMINISTRATION TO S.G., A 21 YEAR OLD PATIENT WITH GONADAL DYSGENESIS. The administration of 2.5 mg HGH was without measurable effect; administration of 10 mg daily caused slight but definite nitrogen, phosphorus, sodium and potassium retention and rise in urinary calcium. Lot 4 HGH was used on Days 6 through 16; Lot 5 was used thereafter.

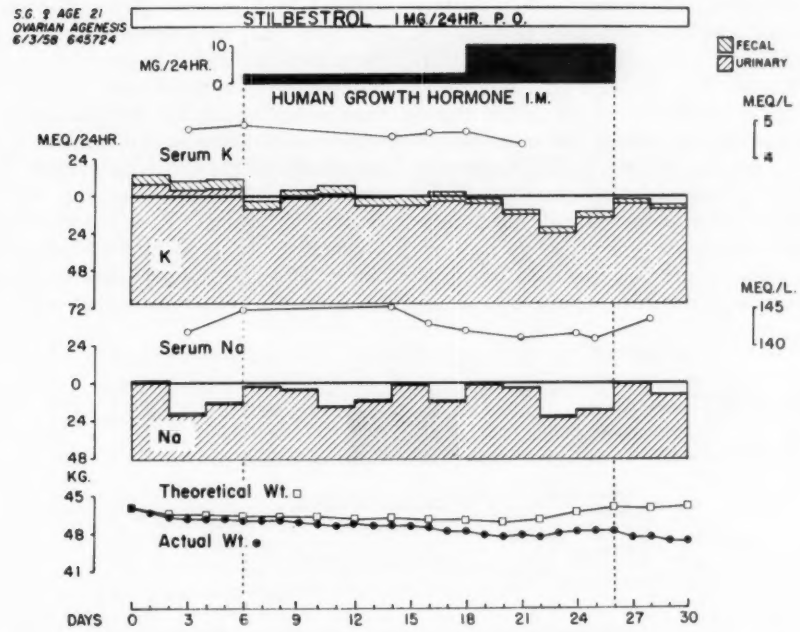

FIG. 6B. POTASSIUM AND SODIUM BALANCE, AND ACTUAL AND THEORETICAL WEIGHTS DURING HGH ADMINISTRATION TO S.G.

ment for growth is met, the rate of nitrogen retention falls to the low rate characteristic of normally growing children (19). The observation in B.D. that a second course of HGH, following 12 days off HGH, again produced very rapid nitrogen retention is unexpected and suggests restoration of some factor(s) when HGH was withdrawn which permits rapid nitrogen retention of brief duration. The phenomenon of early waning of nitrogen retention is probably unrelated to the "plateauing" of growth on more prolonged treatment as recorded in Table II and as reported previously in rats (20). Dr. Jacob Lerman was unable to demonstrate significant serum precipitins to HGH following treatment in two of the patients in this series (18). Restoration of responsiveness occurred in B.D. after 12 days off HGH; increasing or maintained resistance after two weeks would be expected if the mechanism of resistance were the formation of neutralizing antibodies. The waning responsiveness to HGH on prolonged administration raises the possibility that endogenous growth hormone secretion may be cyclical and interrupted rather than continuous. The cement lines of bone are thought to reflect such regular cyclical cessation of growth. The opinion is commonly expressed, although unsupported, that normal children grow in spurts interrupted by phases of fat accumulation.

Calcium retention was greater following rather than during HGH treatment. Does this mean that HGH facilitates calcium absorption and chondrogenesis but that osteogenesis and calcium deposition occur only during periods of little or no growth hormone secretion? The hypercalciuria observed during HGH administration apparently reflects greater stimulation of calcium absorption than of calcium deposition (osteogenesis). It has not been emphasized previously that growth hormone increases calcium absorption; increased calcium absorption was prolonged and did not wane within the 12 to 18 days of observation after stopping HGH.

In addition to potassium retention proportional

to and coincidental with nitrogen retention, there was observed additional potassium retention the first day on HGH and similar disproportional loss of potassium the first two days off HGH. This phenomenon is reminiscent of the similar disproportional loss and retention of potassium the first day on and the first day off ACTH, respectively (21). Since growth hormone has anabolic effects opposite in direction to the antianabolic effects of ACTH this unaccounted-for potassium may represent a tissue or process uniquely affected, although in opposite directions, by the anabolic and antianabolic hormones. Batts, Bennett, Garcia and Stein (22) have demonstrated that a part of the potassium retained on growth hormone is accounted for by a rise in concentration of potassium within muscle and that part of the sodium retained contributes to an expansion of extracellular fluid volume. It is of interest that HGH promotes a simultaneous decrease in urinary sodium and potassium. Some sodium retention was produced by HGH in E.S. (Addison's disease). Since the degree and type (mineralocorticoid or glucocorticoid) of adrenocortical insufficiency may

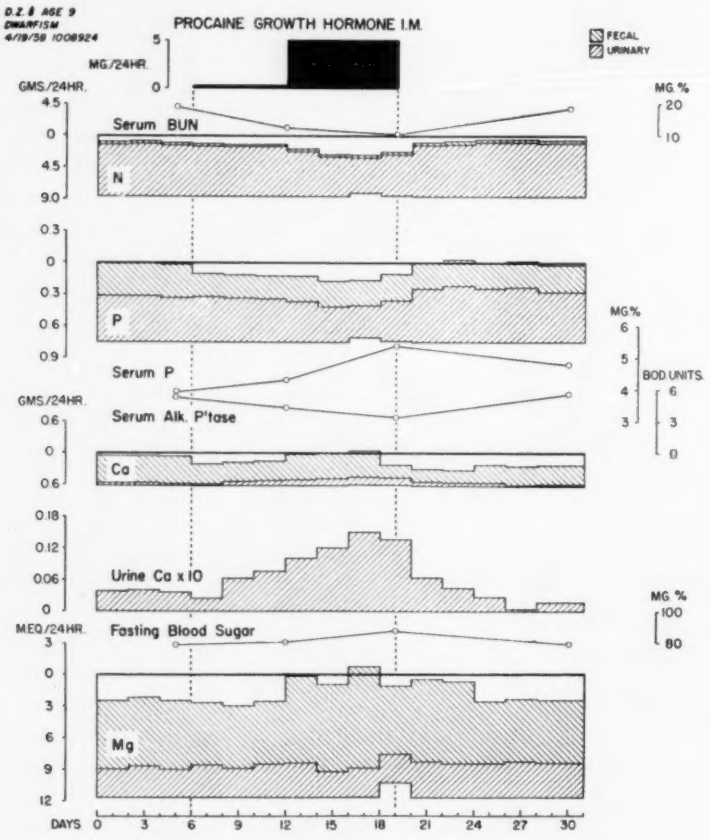

FIG. 7A. EFFECTS OF HGH IN D.Z., A NINE YEAR OLD BOY WITH SHORT STATURE AND DELAYED "BONE AGE." Initial doses of 0.2 mg procaine-HGH daily produced nitrogen, phosphorus, sodium and potassium retention and rise in urinary calcium less marked than that produced by 5 mg daily. Lot 4 HGH was used on Days 6 through 14; Lot 2 HGH was used thereafter.

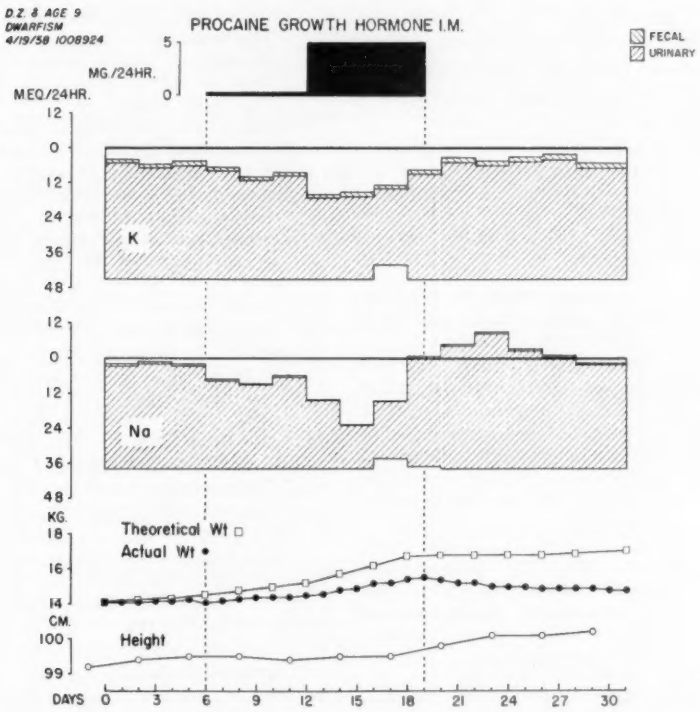

FIG. 7B. POTASSIUM AND SODIUM BALANCES, HEIGHT, AND ACTUAL AND THEORETICAL WEIGHTS DURING HGH ADMINISTRATION TO D.Z.

be incomplete in patients with spontaneous Addison's disease, such sodium retention may have been the consequence of HGH stimulation of aldosterone secretion, as suggested by Beck and colleagues (1) or may indicate a direct renal action of HGH itself.

Prolonged HGH therapy in S.M. was attended by regrowth of a craniopharyngioma which apparently regressed on stopping HGH (see legend of Figure 1A). After 30 days' HGH administration M.A. developed evidence of third ventricle block which was attributed to further growth of her known craniopharyngioma. Further growth hormone therapy was withheld, a Torkildsen procedure for relief of internal hydrocephalus was carried out by Dr. J. C. White, and the patient has since continued in apparent good health for 18 months. Due to the irregular growth rate of craniopharyngiomas it is unclear whether HGH therapy really stimulated growth of these tumors. It is to be noted that partial removal of these tumors often inhibits their further significant growth. This effect might equally well be attributed to the hypopituitarism (and loss of growth hormone secretion) which commonly follows pituitary surgery.

SUMMARY

Daily intramuscular injections of 0.2 to 10 mg daily of the Raben preparation of human growth hormone in 10 patients stimulated linear growth, produced retention of all cellular constituents measured, increased calcium absorption, and probably mobilized fat. At the dose levels used the chemical response to human growth hormone waned after 3 to 4 weeks' continuous treatment; responsiveness was restored by 12 days off growth hormone. As used in this study the Raben prepara-

tion of human growth hormone was stable, well tolerated, apparently free of significant gonadotropic, thyrotropic and adrenocorticotropic contamination, and did not stimulate precipitating or blocking antibodies.

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EFFECTS OF HUMAN GROWTH HORMONE ON LEVELS OF BLOOD AND URINARY CARBOHYDRATE AND FAT METABOLITES IN MAN *

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In an accompanying paper (1) human growth hormone (HGH) was reported to have stimulated the rate of linear growth, produced retention of nitrogen, phosphorus, magnesium, calcium, sodium and potassium, and perhaps increased the rate of fat mobilization in ten human subjects. This paper describes the response of blood and urinary levels of fat and carbohydrate intermediary metabolites in seven of these and in one infant with hyperinsulinism.[1]

Some effects of HGH on carbohydrate and fat metabolism have already been published. Raben and Hollenberg (2) observed a prompt and significant rise in plasma free fatty acids (FFA). Beck, McGarry, Dyrenfurth and Venning (3) reported alterations in glucose tolerance in four of six patients and Ikkos, Luft and Gemzell (4) in two of two patients. Ikkos and associates also observed elevations in ketone bodies in three of four patients. The data to be reported in general confirm these observations and include the simultaneous measurements of blood and urinary glucose, lactate, pyruvate, citrate, α-ketoglutarate, free fatty acids and ketones during short and long-term HGH therapy.

MATERIAL AND METHODS

The clinical features of the patients studied are reported elsewhere (1). The subjects include 5 patients with hypopituitarism, 1 patient with obesity on an 800 calorie diet and 1 patient with proven hyperinsulinism (5). All patients were studied under constant conditions.

Blood levels of carbohydrate and fat intermediary metabolites were measured 4, 8 and 24 hours after injections of 1.0 to 10.0 mg of acid-soluble HGH (Raben).[2] HGH was administered as a single intramuscular injection at 6 a.m. (or beginning of "metabolic day") daily for 2 to 36 days.

The methods for measurement of blood and urinary glucose, lactic, pyruvic, α-ketoglutaric and citric acids, acetone and total ketones, and inorganic phosphorus have been reported previously (6). In addition, plasma FFA were measured by the method of Davis (7) as modified by Gordon and Cherkes (8). Balance studies were conducted according to the method of Reifenstein, Albright and Wells (9).

RESULTS

HGH (1) produced a decrease in blood urea nitrogen (BUN) and urinary nitrogen in 24 to 48 hours which was not apparent within the first four hours after injection. With constant daily HGH injections, nitrogen retention increased for 3 to 5 days, was maximal for 2 weeks, and then decreased to low levels in the fourth and fifth weeks of treatment. If HGH were discontinued for 12 days and then resumed, nitrogen retention was again marked. Other constituents of protoplasm were retained in the relative concentrations characteristic of most tissues.

Because of the multiphasic nature of the nitrogen response to HGH the measurements of intermediary metabolites have been separated as follows: the early phase (4 hours to 3 days), as nitrogen retention is developing; the second phase (3 to 15 days) during sustained maximal nitrogen

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[1] Studied through the courtesy of Dr. John Crigler, Children's Medical Center, Boston, Mass. A second partial pancreatectomy completely alleviated the patient's hypoglycemia.

[2] Similar responses were obtained with a procaine-hydrochloride precipitate of HGH (Raben) (1). This preparation of procaine was epinephrine-free.

TABLE I

*Early effects of HGH on levels of plasma FFA, serum citrate and blood glucose**

Patient	HGH treatment	Day	FFA, plasma Hours after HGH				Citrate, serum Hours after HGH				Glucose, blood Hours after HGH			
			0	4	24	48	0	4	24	48	0	4	24	48
	mg/day		*mEq/L*				*mg/100 ml*				*mg/100 ml*			
M.C.		0	0.30				1.75				74			
	2.5	1	0.15		0.32		2.08		2.50					
		4			0.32				2.41				64	
J.C.	5.0	1	0.63	1.31			3.58	3.0			124	100		
	5.0	2		1.85	1.08	0.53		3.83	4.50	1.0		100	101	72
	5.0	1	0.71	1.20	0.70	0.71	1.92†	4.70	2.7	1.25	78	50	75	74
L.C.		0	0.80											
	5.0	1	0.95	1.05			3.65	3.70			83	84		
		2		1.45	0.94	0.78		3.83	3.2	2.50		115	78	86
						0.82‡				2.53‡				88‡
B.D.	2.5	1	0.74		0.69	0.69	3.60		4.5		74		47	
	5.0	1							5.0	3.42			81	73
	5.0	1	0.67	0.91	0.95		2.82	3.86	4.4		83	74		
	5.0	2			0.65				4.5	3.4			95	79

* All blood samples (from antecubital vein) were obtained after a 12 to 15 hour fast; HGH was administered 4 hours before the blood sample. M.C., J.C. and L.C. were hypopituitary patients, and B.D. was an obese subject on an 800 calorie diet. Blood levels of pyruvate, lactate, ketones and α-ketoglutarate were not significantly altered during this early phase.

† This value may reflect a depression subsequent to the administration of cortisone two days before (10).

‡ Value 72 hours after last dose of HGH.

retention; and the third phase (15 to 36 days), as nitrogen retention declines to low levels.

I. The early phase (4 hours to 3 days)

1. Plasma FFA. Fasting concentrations of FFA were increased 4 hours after each of several injections of HGH in two patients with hypopituitarism and in the obese patient (Table I). Levels were elevated 8 to 12 hours after HGH in the infant with hyperinsulinism (Table II). Levels were not elevated 24 hours after the first dose of HGH, but were elevated 24 hours following the second dose of HGH in one of three subjects (Table I).

2. Serum citric acid. Fasting concentrations of serum citric acid were definitely increased 4 to 8 hours after HGH in three of five observations (J. C., second course; B.D. following 5.0 mg HGH; and P.H; see Tables I and II). Levels were elevated 24 hours after HGH in four of five subjects. Concentrations fell below control in all patients upon discontinuing HGH (Tables I and II) during this phase.

3. Twenty-four hour urinary citrate, acetone and total ketones. Control urinary excretion of citric acid in the five patients with hypopituitarism was 283 mg per 24 hours, a level which is significantly less ($p < 0.001$) than normal (mean of normal subjects on constant diet was 480 mg; SE 28 mg). Daily urinary acetone and ketone levels were within normal limits during the con-

TABLE II

*Effects of HGH on plasma FFA, serum citrate and blood glucose in an infant with hyperinsulinism**

HGH treatment	Day of treatment	Hours after last dose	FFA	Citrate	Glucose
			mEq/L	*mg/100 ml*	*mg/100 ml*
1.0 mg/12 hours for 6 days	0		0.36	1.60	48
	(Control)		0.45	1.40	
	1	8	1.16	2.83	29
	1	12	1.16		24
	6	12	1.36	1.83	24
2.5 mg/day for 6 days	7	8	1.36	2.83	25
	10	8	1.36	2.26	
	12	24	0.78	1.16	20
Postcontrol	6		0.56		
	12		0.46	0.3	22

* All samples were taken after a 4 to 8 hour fast. The patient was fed every 8 hours. Glucose levels were measured every 3 to 4 hours. Values listed for control represent mean of a 6 day period prior to HGH. Patient received HGH for 12 consecutive days and showed a significant increase above control in nitrogen retention during this period (5).

trol period. Urinary excretion of citrate, acetone and ketones increased significantly ($p < 0.01$) during the first 24 hours after the injection of 1.0, 2.5 or 5.0 mg of HGH in all patients studied except the patient with obesity (Table III). In the patients with hypopituitarism, mean citrate excretion rose from 283 (SE 20 mg) to 362 mg (SE 17 mg); mean acetone from 2.1 (SE 0.5 mg) to 7.5 mg (SE 1.7 mg); and mean ketones from 15 (SE 3.3 mg) to 32 mg per 24 hours (SE 5.4 mg). In Patient S.M., urinary acetone, ketone and citrate increased further with each 1 mg injection of HGH administered every third day (Table III); the degree of rise in ketones was greater than that in citrate. In the patient with obesity (B.D., Figure 1 and Table III) urinary citrate and ketones fell during the first 24 to 72 hours of HGH administration.

II. The second phase (3 to 15 days)

1. Plasma FFA. Fasting levels of FFA (24 hours after each injection of HGH) rose above control by the sixth day of HGH (2.5 mg) in the one patient with hypopituitarism so tested (M.C., Figure 2), were not increased on the sixth day but were on the fourteenth day of HGH (2.5 mg per day) in the patient with obesity (B. D., Figure 1), and were elevated in the infant with hyperinsulinism on the sixth, seventh, tenth and twelfth days of HGH (Table II). Once elevated, FFA concentrations generally remained above control during the first two weeks of therapy (Figure 2, Table II).

2. Serum and urinary citric acid. During the first two weeks of daily HGH treatment, fasting serum and/or urinary citrate rose in five patients (B.D., Figure 1; M.C., Figure 2; P.H., Table II; M.A., Table IV; and S.M., Table V). Serum levels were not elevated in one of the patients with hypopituitarism (M.A., Table IV) on the twelfth day of HGH administration at a time when nitrogen retention was still marked; however, urinary citrate excretion on this day was 260 mg above control.

In the obese patient, B.D., the initial fall in urinary citrate during Phase I of HGH was followed by a gradual rise to levels above control throughout the period of maximal nitrogen retention. Increasing the dose of HGH from 2.5 to 5.0 mg produced a second transient decrease in citrate excretion to a low of 379 mg per 24 hours on the third day (Days 66 to 72, charted as two

TABLE III

*Early effects of HGH on urinary metabolites**

		Acetone				Ketones				Citrate			
			Day of treatment				Day of treatment				Day of treatment		
Patient	Dose HGH	Control	1	2	3	Control	1	2	3	Control	1	2	3
			mg/24 hrs				*mg/24 hrs*				*mg/24 hrs*		
M.C.	2.5 mg/day	4.0	3.6	4.1	3.7	21.6	24.0	21.2		214	325	320	338
S.M.	1 mg/3 days	1.7	6.0	6.9	2.0	4.7	20.8	20.8	18.2	280	307	307	290
	1 mg/3 days		4.5	9.2	4.0		45.3				330	330	297
	1 mg/3 days		3.9		3.8		12.5		12.5		357	249	
	1 mg/3 days		2.3	12.0	11.0		62.3	62.3	62.3		249	340	340
M.A.	2.5 mg/day	2.1	11.1	9.1	10.0	17.5	37.5	39.8	21.8	330	400	400	466
L.C.	5 mg/day					24.0	40.0	15.3		325	486	295	
J.C.	I 5 mg/day	0.4	0.5	3.9		16.8		21.9		321	370	296	
	II 5 mg/day	0.9	6.0			24.5	34.0			245†	328		
B.D.	2.5 mg/day	12.0	19.4	17.2	15.8	58.0	34.3	65.3	65.3	555	535	320	320

* All but B.D. were hypopituitary patients. B.D. was an obese subject on an 800 calorie diet. S.M. received single injections of 1 mg HGH every 3 days; values listed were obtained on the first, second and third days after each of these injections. J.C. study was as follows: fore-control (3 days); HGH 5 mg per day (2 days); postcontrol (3 days); cortisone 10 mg per day (2 days); postcontrol (3 days); and finally HGH 5 mg per day (1 day). No significant changes in urinary pyruvate or lactate were observed.

† Values, second control, immediately followed cortisone administration which would be expected to lower urinary citrate (10).

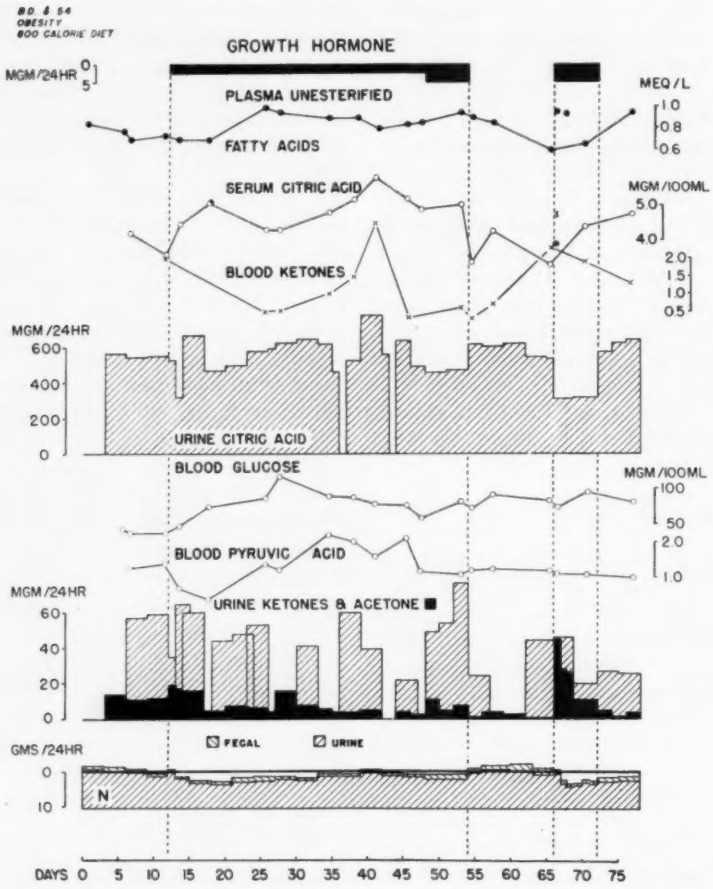

FIG. 1. EFFECTS OF HGH DURING CALORIC RESTRICTION OF AN OBESE PATIENT (B.D.). The patient was on an 800 calorie constant diet for 12 days prior to and during the entire study. Her initial weight was 440 pounds. All blood samples were obtained in the fasting state 24 hours after the last dose of HGH except on Days 66 and 67 when blood samples were obtained 4 hours after HGH (values plotted separately). Urinary citrate, acetone and ketones were not measured on the days where no values are charted. Overlapping of lines in urinary ketones represents measurements made on aliquots of different 3-day pools of urine. Plasma unesterified fatty acids = free fatty acids (FFA).

3-day pools in Figure 1). Nitrogen retention at this time was considerably more than that observed during the first course of 5 mg HGH per day.

3. Blood α-ketoglutaric acid. Fasting concentrations of α-ketoglutaric acid increased from 0.12 to 0.47 mg per 100 ml by the sixth day of HGH (2.5 mg per day in B.D., the patient with obesity). Levels remained above control and abnormally high during the first two weeks of HGH in this patient.

4. Glucose metabolism. Fasting concentrations of glucose progressively decreased in Patient M.C. (Figure 2) and increased in the patient with obesity (B.D., Figure 1). There was no significant change on the twelfth day of treatment in

TABLE IV

*Effect of prolonged HGH therapy in hypopituitarism**

Treatment	Duration	Venous blood levels					Urinary excretion			
							Citrate		Ketones	
		Glucose	Pyruvate	Lactate	Citrate	Ketones	Mean	SE	Mean	SE
	days	*mg/100 ml*					*mg/24 hrs*			
Fore-control	12	60	1.4	7.5	2.8	0.68	330	16.9	17.5	0.8
HGH 2.5 mg/day	12 (Day 3–15)	69	1.7	8.0	2.5	0.44	531	5.4	19.3	3.3
HGH 2.5 mg/day	15 (Day 15–30)	68	1.6	6.5	2.13	0.75	545	20.0	21.5	1.0

* Patient M.A.; fasting, 10 a.m. blood samples were drawn on last day of designated period, and 4 hours after daily administration of HGH. Cortisone, 10 mg daily, was administered throughout the study. Values for urinary levels represent mean excretion during entire designated period. Urinary levels of pyruvate and lactate showed no significant change. SE = standard error of the mean.

Patient M.A., but glucose tolerance was decreased at this time: *1*) Control test: fasting, 60; 1 hour past glucose, 68; and 2 hours past glucose, 54 mg per 100 ml. *2*) After 12 days of HGH: fasting, 69; 1 hour past glucose, 123; and 2 hours past glucose, 105 mg per 100 ml.

5. Twenty-four hour urinary excretion of ketones. During the period of maximal nitrogen retention, urinary ketones fluctuated but were generally above control in the three patients with hypopituitarism. In M.A. and S.M. (Tables IV and V) cortisone appeared to decrease the degree of ketonuria relative to the rise in citrate. Withdrawal of cortisone in S.M. was followed by an increase in ketonuria (Table V).

Due to caloric restriction, fore-control levels of plasma FFA (0.74 mEq per L), serum citrate (3.6 mg per 100 ml), urinary ketones (60 mg per 24 hours) and urinary citrate (555 mg per 24 hours) were all abnormally elevated in B.D. prior to HGH. Urinary acetone and blood and urinary ketones fell to normal levels during nitrogen retention (Figure 1).

III. The third phase (15 to 36 days)

By the end of the fourth week, daily administration of HGH in the hypopituitary patients resulted in waning nitrogen retention (1) and similar declines toward control in levels of plasma FFA, and serum and urinary citrate (Figures 1

TABLE V

*Effects of prolonged HGH therapy in hypopituitarism**

Treatment	Duration	Venous blood levels					Urinary excretion			
							Citrate		Ketones	
		Glucose	Pyruvate	Lactate	Citrate	Ketones	Mean	SE	Mean	SE
	days	*mg/100 ml*					*mg/24 hrs*		*mg/24 hrs*	
Fore-control	9	72	1.1	8.3	3.16	1.0	280	6.9	4.7	0.2
HGH 1 mg/3 days	12						325	8.3	22	5.9
HGH 1 mg/day	18	62	1.7	5.4	3.42	0.5	349	8.5	35	5.3
HGH 1 mg/day	12						343	11.3	13	1.7
Cortisone 10 mg/day										
HGH 5 mg/day	12						380	25.9	17	1.7
Cortisone 10 mg/day										
Cortisone 10 mg/day	18	65	0.9	5.7	1.20		224	13	7.5	0.7
Postcontrol†	1–6(a)	69	1.1	9.1	2.3	0.5	234	12.4	26.5	0.8
	7–12(b)								7.0	0.9

* Patient S.M.; fasting 10 a.m. blood levels were drawn on last day of designated periods, and 4 hours after medication. Urinary excretions represent mean of all observations of entire control or treatment period. Urinary levels of lactate and pyruvate showed no significant change.

† Postcontrol period with no treatment is divided into the first 6 days (a) and the second 6 days (b) for ketone values. No significant difference in other urinary metabolites was observed between the first and second 6 day postcontrol periods.

and 2). In B.D., waning nitrogen retention was accompanied by a decline in plasma FFA and blood α-ketoglutaric acid (0.22 mg per 100 ml on Day 45 of study). Urinary citrate and ketones were at or below control in B.D. during the fifth week of treatment.

During the third and fourth weeks of therapy, fasting concentrations of blood glucose were below control levels in S.M. (Table V) and M.C. (Figure 2) and were falling toward control levels in B.D. (Figure 1). Fasting concentrations of blood pyruvate were increased (Figures 1, 2 and 3) while those of lactate were decreased (Figures 2 and 3 and Tables IV and V) in four patients so studied.

Decreased glucose tolerance was observed in the three patients with hypopituitarism. Data from M.C. are shown in Figure 3. In S.M., after 30 days of HGH, glucose concentrations were: fasting, 62; 1 hour past glucose, 171; and 2 hours after glucose, 198 mg per 100 ml. In

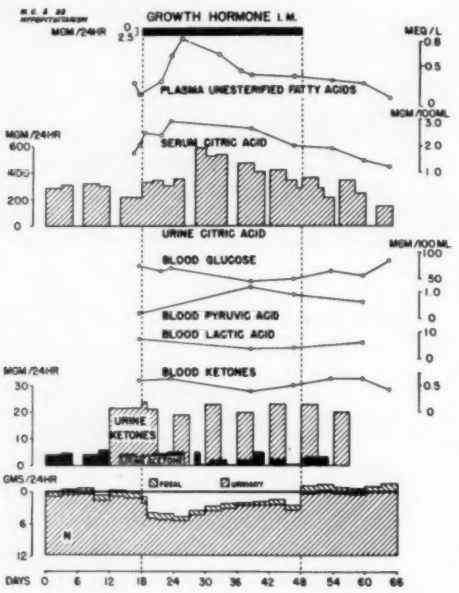

FIG. 2. EFFECTS OF PROLONGED HGH ADMINISTRATION IN M.C. (HYPOPITUITARISM). No measurements of urinary citrate, acetone and ketones were made on the days when no values are shown. Urinary lactate and pyruvate were determined throughout the study and showed no significant change. Blood samples were taken as indicated in legend for Figure 1.

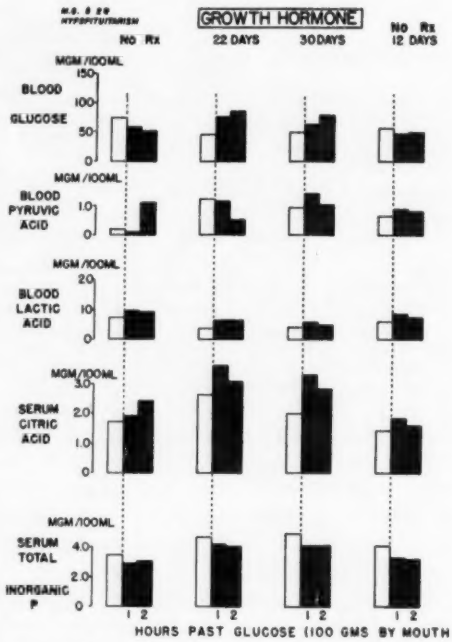

FIG. 3. EFFECTS OF HGH ON RESPONSE TO GLUCOSE IN M.C. (HYPOPITUITARISM). Glucose (100 g) was administered by mouth after a 15 hour fast. Blood samples were taken before, 1 and 2 hours after the ingestion of the glucose. Open blocks represent fasting samples; solid blocks represent postglucose samples.

M.A., after 30 days of HGH, glucose concentrations were actually lower than those observed after 12 days of HGH: fasting, 68; 1 hour after glucose, 100; and 2 hours past glucose, 86 mg per 100 ml.

When HGH was discontinued in the patients with hypopituitarism, urinary citrate and ketones fell to or below control (Figure 2 and Table V).

DISCUSSION

Human growth hormone in man produces consistent changes in serum and urinary citrate and ketones, plasma free fatty acids and glucose tolerance. Elevations in plasma FFA and serum and urinary citrate generally preceded detectable changes in nitrogen metabolism. As suggested by studies in small animals (11) these changes may reflect primary fat mobilization which would increase the supply of two-carbon fragments for

subsequent disposal via the tricarboxylic acid cycle and thereby provide energy and substrate for subsequent protein synthesis. It is of special interest that B.D., an obese patient who was mobilizing fat due to caloric restriction, demonstrated similar elevations in plasma FFA and serum and urinary citrate prior to HGH. The nitrogen balance data (1) suggest a one to three day delay in establishment of maximal nitrogen retention and a waning of such maximal retention after three weeks' continuous treatment. Changes in levels of blood and urinary metabolites showed similar time relations. Thus it appears that the early metabolic response to HGH may not be identical with the response to more prolonged administration of the hormone. The relation of these changes to rate and magnitude of changes in various pathways of cellular intermediary metabolism is uncertain at present.

SUMMARY

Administration of human growth hormone (Raben) to five patients with hypopituitarism, one with hyperinsulinism, and one with obesity produced alterations in blood levels of carbohydrate and fat metabolites which preceded marked detectable changes in protein metabolism by approximately 24 hours. There was a prompt rise in plasma free fatty acids and blood and urinary citrate and ketones.

Continued daily doses of human growth hormone produced marked nitrogen retention, glucose intolerance, a sustained rise in plasma free fatty acids and serum and urinary citrate, elevations in blood pyruvate and a decrease in blood lactate. Administration of human growth hormone for more than two to three weeks resulted in gradual return of nitrogen excretion and blood and urinary metabolites toward control levels but continued glucose intolerance.

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THE TOXIC EFFECTS OF CARBON DIOXIDE AND ACETAZOLAMIDE IN HEPATIC ENCEPHALOPATHY *

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Striking alterations in respiratory gas and blood ammonia levels usually accompany hepatic coma. The arterial pH is elevated and the carbon dioxide tension reduced (1). The blood ammonia level (2–4) is increased in many instances and the brain arteriovenous ammonia difference is high (5, 6). The cerebral oxygen uptake is depressed (7, 8).

Whether the pronounced alkalosis which accompanies many instances of hepatic coma contributes to the encephalopathy is unknown. Severe blood alkalosis impairs oxyhemoglobin dissociation (9), lowers the arterial oxygen partial pressure and interferes with cerebral function in normal subjects (10). Alkalosis is reported to enhance ammonia toxicity (11–13). These effects led Roberts and associates (14) as well as others (13) to suggest using carbon dioxide inhalation to treat hepatic coma. Preliminary clinical observations on our own services suggested that inhaled CO_2 worsened rather than improved patients with the encephalopathy of liver disease. Accordingly, detailed clinical and cerebral metabolic studies were carried out to quantitate the effects on brain function of raising blood and tissue CO_2 tensions. The results of the study are reported here. Cerebral blood flow and cerebral oxygen uptake were measured at rest in control and cirrhotic patients and the results were correlated with the patients' mental and somatic neurological function. Following the baseline study, selected patients inhaled carbon dioxide and the clinical cerebral metabolic observations were repeated. Subsequently, to eliminate the undesirable side effects of hyperpnea and dyspnea as contributing to the observed changes, similar observations were made before and after raising tissue CO_2 tensions by rapidly infusing acetazolamide (Diamox).

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MATERIALS, PROCEDURE AND METHODS

Materials

A total of 17 patients with hepatic cirrhosis diagnosed by the usual clinical and laboratory criteria were selected from the wards and clinics of the King County Hospital, Seattle. Eleven patients of comparable age, lacking cirrhosis or other diseases known to affect cerebral metabolism, served as "normal" controls. Four noncirrhotic patients with old clinically-stable brain lesions (one with focal brain infarction, two with diffuse, moderate cerebral arteriosclerosis, and one with post-hypoglycemic brain damage) served as controls for studying the effect of CO_2 inhalation on cerebral metabolism. One of these subjects and four additional subjects with cerebral arteriosclerosis served as controls when the effects of acetazolamide on hepatic encephalopathy were studied. All patients and controls received careful mental and neurological examinations. The degree of encephalopathy in the cirrhotics was graded from 0 to 4 using the following criteria.

Grade 0. The patients were fully alert and oriented. They had intact recent memory and performed serial sevens or serial threes correctly and easily. The somatic neurological examination was normal. The respiratory rate and depth appeared normal.

Grade 1. The patients were fully alert and oriented. Although recent memory was good, mental testing uncovered errors, e.g., date of admission to hospital. Serial sevens were mildly impaired. The somatic neurological examination was normal. The respiratory rate and depth appeared normal. Since presumably normal persons from a similar age group and socioeconomic background frequently show this minimal impairment, Grade 1 was considered uncertain evidence for encephalopathy and was clinically classified with Grade 0 for metabolic analysis.

Grade 2. The patients were lethargic and mildly confused. They usually were oriented correctly to person and place but incorrectly to time. Serial sevens were performed poorly. Most patients had arrhythmic lateral twitching of the extended fingers and some had the classic "metabolic flap" described by Adams and Foley (15). The respiratory rate and depth appeared normal.

Grade 3. The patients were frankly confused and intermittently somnolent. Disorientation was usually complete so that appropriate conversation was impossible. The somatic neurological examination often elicited hy-

peractive deep tendon reflexes, paratonic rigidity and extensor plantar responses. A metabolic flap was invariably present.

Grade 4. The patients were comatose and responded only to vigorous painful stimuli. They were obviously hyperventilating.

Procedure

All patients were taken to the laboratory in the early morning before breakfast. A mental and somatic neurological examination was performed. Following this, no. 18 needles were inserted into the femoral artery and jugular bulb (16), and a snugly fitting face mask was applied. The blood pressure and alveolar CO_2 tension (P_{ACO_2}) were monitored. The patients rested for 20 minutes or more while respiratory exchange and blood pressure stabilized. Cerebral blood flow using 15 per cent N_2O, 21 per cent O_2, and 64 per cent N_2 was then determined. During the determination each patient was observed repeatedly for variations in muscular tremor and resistance. At the end of this first procedure, all patients rested 10 to 15 minutes. Following this rest period, the effects of inhaled CO_2 or infused acetazolamide were then studied in selected subjects.

To determine the effects of inhaled CO_2, subjects were placed on 5 per cent CO_2 in air for approximately 20 minutes to develop a stable response to the gas. After this adaptation, the gas mixture was switched to 15 per cent N_2O, 5 per cent CO_2, 21 per cent O_2 and 59 per cent N_2, and the cerebral blood flow was repeated, muscular tremor and resistance being watched closely throughout. Detailed mental and neurological testing was repeated as soon as CO_2 breathing terminated.

To determine the effects of acetazolamide, 0.5 to 2 g of the drug was infused intravenously, several minutes being allowed for administration. Thirty minutes later the cerebral blood flow determination was repeated, with the mental and somatic neurological examination being repeated at the end of this second procedure.

Methods

The cerebral blood flow was determined by Scheinberg and Stead's modification (17) of Kety and Schmidt's original method (18). Slight variations improved the technique's accuracy (19). The 10-minute samples were machine-drawn at a constant rate to assure more accurate integration. Calculations (17) included arithmetical correction for dead space between vessel and syringe (19).

Blood was collected in heparin-washed syringes, immediately capped and mercury-sealed. Care was taken to avoid air bubbles. Immediately after drawing, the blood pH was determined at 37° C with a Beckman microelectrode attached to a Cambridge model R pH meter. Each sample was repeatedly measured until duplicate agreement of 0.01 pH unit was achieved. The duplicate blood oxygen and carbon dioxide contents were measured manometrically by Peters and Van Slyke's method (20), and the arterial carbon dioxide tension (P_{aCO_2}) was determined on the Singer and Hastings nomogram (21). Oxygen tension was estimated from the Dill nomogram, using Severinghaus' correction factors for pH (22). The blood nitrous oxide was measured by Kety and Schmidt's method (18), each sample being repeated until duplicate agreement of 0.02 volume per cent was achieved. The blood ammonia was determined by Seligson and Hirahara's method (23) and the mean of the two closest of three determinations was the figure chosen.

The arterial blood pressure was recorded directly from the femoral artery through a Statham transducer and a Sanborn strain gage amplifier. The expired carbon dioxide was monitored by an infrared analyzer (Liston-Becker), gas samples being drawn continuously through a needle in the patient's mask.

RESULTS

Normal values

Table I lists the mean respiratory gas and cerebral metabolic values of eleven "normal" control patients. The mean arterial blood pH (7.42) and Pa_{CO_2} (38 mm Hg) were similar to the values of other investigators (22). The mean cerebral blood flow (CBF, 53 ml per 100 g per minute), arteriovenous oxygen difference (A–V, 6.6 volumes per cent) and cerebral metabolic rate for oxygen (CMR_{O_2}, 3.4 cc per 100 g per minute) were virtually identical with Kety and Schmidt's data on normal subjects (18) but significantly lower than Scheinberg and Stead's (17). To check our methods, each of three patients with static central nervous system disease had cerebral blood flows determined on two different days. The CBF and $A–V_{O_2}$ differences varied considerably between determinations, but the CMR_{O_2} showed a standard deviation of 0.182, a value similar to Kety and Schmidt's 0.187.

Cerebral metabolism in cirrhosis

Blood pH. Table I and Figure 1 show the arterial pH levels from cirrhotic patients in Grades 0 to 4. There was overlapping between individual pH values accompanying different grades of encephalopathy although patients with moderate and severe encephalopathy had higher mean pH determinations than did patients graded 0 to 2. All but one of the patients without encephalopathy were alkalotic (mean pH 7.48), although clinically evident hyperventilation was absent. Most Grade 3 patients had higher arterial blood

TABLE I

Cerebral metabolism in cirrhosis

Name, Age, Sex	Art. pH	Art. O_2	Est. P_{O_2}	P_{aCO_2}	Cerebral A-V_{O_2}	CBF*	CMR_{O_2}†
		Sat. %	*mm Hg*	*mm Hg*	*vol %*	*ml/100 g/min*	*cc/100 g/min*
Mean, 11 normals	7.42±0.03	95.2±2.0	89	38±5	6.61±1.09	53±8	3.4±0.6
Grade 0							
V.McL. 42♀	7.47	91.8	62	29	4.83	70	3.4
M.R. 49♂	7.42	90.9	61	30	5.13	64	3.3
G.B. 50♂	7.51	86.1	48	35	5.49	51	2.8
Grade 1							
A.A. 47♀	7.52	92.9	61	30	6.85	39	2.7
E.deS. 58♂	7.46	87.0	52	38	6.31	72	4.5
F.W. 48♂	7.48	91.5	60	38	6.54	55	3.6
Mean, Grade 0–1	7.48±0.03	90.0±2.5	57±5.3	33±4	5.86±0.75	59±12	3.4±0.6
Grade 2							
L.J. 68♂	7.47	94.3	71	27	7.54	43	3.2
G.L. 72♂	7.43	92.5	66	33	6.76	43	2.9
A.G. 63♂	7.49			36	5.47	51	2.8
L.J. 33♂	7.45	93.5	68	27	4.67	51	2.4
C.K. 50♂	7.52	92.0	59	36	6.63	45	2.9
C.K. 50♂	7.49	93.5	65	28	5.05	59	3.0
M.P. 65♀	7.47	91.3	61	24.5	6.19	50	3.1
H.D. 59♂		94.8			6.97	36	2.5
T.L. 33♀	7.51	91.9	59	22.5	5.20	48	2.5
Mean, Grade 2	7.48±0.03	93.0±1.2	64±4.3	29±5	6.05±0.93	47±6	2.8±0.3 p = <0.01
Grade 3							
A.G. 63♂	7.62	96.0	70	28	6.43	33	2.1
C.K. 50♂	7.53	92.0	58	34	5.85	36	2.1
C.K. 50♂	7.49	93.4	65	32	3.37	35	1.2
F.H. 54♂	7.55	79.1	38	35	6.32	34	2.2
F.H. 54♂	7.62	83.1	39	28	6.00	44	2.6
E.L. 55♂	7.51	92.8	61	24	5.42	32	1.8
Mean, Grade 3	7.55±0.05	89.5±6.1	55±12.3	30±4	5.56±1.15	36±4	2.0±0.4 p = <0.01
Grade 4							
G.R. 63♀	7.63	90.4	49	22	6.15	29	1.8
B.F. 84♀	7.57	93.3	59	27	8.05	20	1.6
B.F. 84♀	7.52	92.3	59	25	7.38	18	1.3
Mean, Grade 4	7.57±0.05	92.0±2.6	56±4.7	25±6	7.19±1.37	22±5	1.6±0.2 p = <0.1 >0.05

* Cerebral blood flow.
† Cerebral metabolic rate for oxygen.

pH values (mean 7.55) than did those in Grades 0, 1 or 2, although only one Grade 3 patient showed clinically evident hyperventilation. Both of the Grade 4 patients (one patient studied twice) had an arterial pH above 7.52, and both were hyperventilating clinically.

Cerebral oxygen uptake. Table I and Figure 1 show CMR_{O_2} values from cirrhotic patients. There was a consistent trend toward decreasing cerebral oxygen uptake as encephalopathy worsened. Patients without definite encephalopathy (Grades 0 and 1) had normal cerebral blood flow and oxygen uptake (mean 3.4 cc per 100 g per minute). Patients with moderate encephalopathy (Grade 2) showed a significant ($p = < 0.01$) CMR_{O_2} depression (mean 2.8 cc per 100 g per minute). The lowered cerebral oxygen uptake was reflected in some Grade 2 subjects by reduced

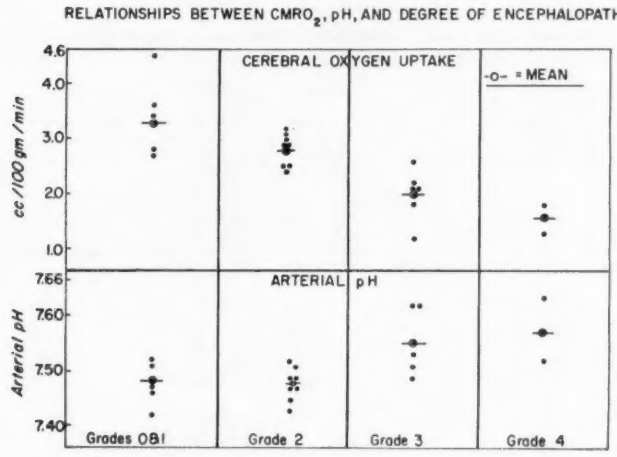

FIG. 1. CEREBRAL OXYGEN UPTAKE AND ARTERIAL pH VALUES FOR PATIENTS WITH HEPATIC ENCEPHALOPATHY, GRADED 0–4.

CBF and in others by reduced $A–V_{O_2}$ differences. All Grade 3 patients had reduced CBF; in none was the cerebral $A–V_{O_2}$ difference raised above normal to compensate for the reduction in flow, and all had reduced CMR_{O_2} values (mean 2.0 cc per 100 g per minute). The lowest CBF's were found in the two comatose patients (Grade 4). $A–V_{O_2}$ differences were mildly increased but not sufficiently to compensate for the strikingly reduced blood flow, and CMR_{O_2} was decreased below 2.0 cc per 100 g per minute (mean 1.6 cc) in each determination.

Effect of carbon dioxide inhalation

Clinical response. One cirrhotic and four Grade 0 patients and four noncirrhotic subjects with static brain lesions showed no mental or neurological alterations after inhaling 5 per cent CO_2 for 30 minutes. By contrast, five of the eight cirrhotic patients (one patient was studied twice) with encephalopathy became clinically worse during and shortly after inhaling 5 per cent CO_2. They showed increased confusion, disorientation and dysarthria, as well as intensified amplitude and frequency of muscular tremor. The mental and neurological deterioration was transient (30 to 120 minutes) in four, but the fifth (L.J.) progressed into coma and died 72 hours later. Paradoxically, L.J. was the only one of the five patients whose clinical deterioration during the second blood flow determination was unaccompanied by a reduced cerebral oxygen uptake.

Metabolic effects. Since the response of cirrhotics without encephalopathy to CO_2 inhalation was similar to that of the controls, data in these groups are summarized together in Table II. Table III lists the cerebral metabolic responses of patients with hepatic encephalopathy. Figure 2 compares the CMR_{O_2} responses in the two groups.

The arterial pH fell and the Pa_{CO_2} rose in every patient during CO_2 inhalation. These pH and Pa_{CO_2} changes were greater when the patient had encephalopathy, indicating a lesser ventilatory response to CO_2. Carbon dioxide inhalation evoked a 33 per cent mean CBF increase among noncirrhotic controls and a 46 per cent mean CBF increase among cirrhotics. Each group had several subjects in whom CBF did not increase significantly. These changes are considerably lower than are normal responses of the cerebral circulation to increased CO_2 (18) and were to be expected among the controls, three of whom were selected because of arteriosclerotic brain damage. The diminished circulatory response of cirrhotics was unexpected, but may, in part, also be explained by arteriosclerosis, since their mean age was 56 years. In any case, the mean CBF response was roughly comparable be-

TABLE II

Cerebral metabolic changes following CO_2 inhalation in controls and patients without encephalopathy

	Art. pH		$Paco_2$		Cerebral A-Vo_2		CBF		$CMRo_2$		A-Vnh_3		$CMRnh_3$	
	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2
			mm Hg		*vol %*		*ml/100 g/min*		*cc/100 g/min*		*μg/ml*		*μg/100 g/min*	
Controls														
G.S. 59 ♀	7.45	7.41	35	39	5.17	4.81	60	65	3.1	3.1				
A.Q. 68 ♂	7.40	7.37	37	39	6.74	3.01	43	101	2.9	3.0				
A.H. 68 ♂	7.39	7.37	44	46	9.01	7.29	39	44	3.5	3.2	1.83–1.68	1.73–1.55	5.85	−0.88
O.M. 57 ♂	7.52	7.49	28	32	4.20	4.89	52	47	2.2	2.3				
Cirrhotics														
G.B. 50 ♂	7.50	7.45	35	41	5.49	4.91	51	55	2.8	2.7				
V.McL. 42 ♀	7.47	7.44	29	32	4.83	3.40	70	124	3.4	4.0				
A.A. 47 ♀	7.52	7.50	30	33	6.85	5.78	39	48	2.7	2.8				
E.dS. 58 ♂	7.46	7.40	38	45	6.31	4.00	72	110	4.5	4.4				
Mean	7.46	7.44	34	38	6.08	4.76	53	74	3.1	3.2				
									$p = >0.6$					

tween the controls and cirrhotics without encephalopathy listed together in Table II, and the subjects with hepatic encephalopathy listed in Table III.

Carbon dioxide inhalation failed to affect the cerebral oxygen uptake significantly either in controls or in those cirrhotics who lacked encephalopathy. The mean resting CMR_{O_2} of controls and subjects without encephalopathy was 3.1 cc per 100 g per minute, and during CO_2 breathing this mean value rose insignificantly to 3.2 cc per 100 g per minute. Only one subject decreased his CMR_{O_2} more than 0.1 cc per 100 g per minute.

The cerebral metabolic response to inhaled CO_2 was quite different in patients with hepatic encephalopathy: six of the eight tested patients showed significant CMR_{O_2} reductions during CO_2 breathing. Thus, the mean resting CMR_{O_2} of patients having hepatic encephalopathy was 2.3 cc per 100 g per minute, and this mean value fell

TABLE III

Cerebral metabolic changes following CO_2 inhalation in patients with hepatic encephalopathy

	Art. pH		$Paco_2$		Cerebral A-Vo_2		CBF		$CMRo_2$		A-Vnh_3		$CMRnh_3$	
	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2	Air	CO_2
			mm Hg		*vol %*		*ml/100 g/min*		*cc/100 g/min*		*μg/ml*		*μg/100 g/min*	
Grade 2														
G.L.	7.45	7.43	33	35	6.76	5.26	43	46	2.9	2.3				
L.J.*	7.47	7.42	27	31	7.54	3.88	43	82	3.2	3.3				
C.K.*	7.53	7.51	34	36	5.85	3.14	36	36	2.0	1.0	2.61–2.29	2.14–1.88	11.52	9.36
Grade 3														
A.G.	7.49	7.49	35	36	5.47	4.49	51	53	2.8	2.4				
F.H.*	7.55	7.46	35	42	6.32	5.35	34	36	2.2	1.9	3.54–3.40	3.38–3.32	5.78	2.16
E.L.*	7.51	7.34	24	38	5.42	1.80	32	70	1.8	1.3				
A.G.*	7.61	7.55	28	34	6.43	3.51	33	43	2.1	1.5				
Grade 4														
B.F.	7.57	7.42	27	40	8.05	3.39	20	45	1.6	1.5	2.40–1.63	1.93–1.78	15.40	6.75
G.R.	7.63	7.38	22	42	6.15	1.70	28	60	1.8	1.0				
Mean	7.53	7.44	29	37	6.44	3.61	36	52	2.3	1.8				
									$p = <0.01$					

* Clinically deteriorated with CO_2.

TABLE IV

Change in cerebral metabolism after acetazolamide in control patients

Name, Age, Sex	Acetazolamide dose	Art. pH		Cerebral A-Vo_2		CBF		$CMRo_2$		A-VNH_3		$CMRNH_3$	
		Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.
	g			*vol %*		*ml/100 g/min*		*cc/100 g/min*		*μg/ml*		*μg/100 ml/min*	
O.M. 57♂	1	7.52	7.52	5.49	3.00	46	80	2.5	2.4				
A.Q. 68♂	2	7.40	7.37	6.74	3.01	43	101	2.9	3.0	2.29–2.05	2.02–2.01	10.3	1.0
E.B. 71♂	2	7.50	7.46	6.08	3.46	38	74	2.3	2.5	2.29–1.98	2.49–1.93	11.8	41.5
H.D. 53♂	2	7.48	7.44	8.15	3.91	34	78	2.8	3.0	1.71–1.88	1.75–1.86	−5.78	−8.58
O.W. 59♂	2	7.45	7.42	5.93	3.13	50	77	3.0	2.3	2.11–1.80	1.77–1.65	15.50	9.24
Mean								2.8	2.7				
								p* = >0.8					

* Values calculated for patients receiving 2 g acetazolamide.

significantly ($p = < 0.01$) to 1.8 cc per 100 g per minute during CO_2 breathing; only two of these eight patients with encephalopathy decreased their CMR_{O_2} less than 0.3 cc per 100 g per minute.

Cerebral ammonia uptake. Four patients had arterial and venous ammonia levels measured while CO_2 responses were being determined (Tables II and III). All showed decreased arterial ammonia levels and decreased cerebral ammonia uptakes (CMR_{NH_3}) during the carbon dioxide inhalation. Two of the four patients had a decreased CMR_{O_2} concomitant with the decreased ammonia uptake.

Effect of acetazolamide infusions

Clinical response. No clinical change was observed during the metabolic testing period in any of the noncirrhotic controls receiving acetazolamide. Later, starting 2 hours after the procedure and persisting for approximately 12 hours, one control patient (H.D.) developed drowsiness, nausea and facial paresthesias. Two cirrhotic patients receiving acetazolamide, 0.5 to 1 g showed no clinical change during or after the procedure.

Five patients [1] with hepatic encephalopathy deteriorated neurologically when given 2 g acetazolamide. Starting 20 to 30 minutes after the injection their lethargy, confusion and disorientation worsened, their dysarthria progressed and their metabolic-type tremor increased in frequency and amplitude. The changes, whose degree was alarming in one subject (T.H.), were temporary and lasted from 6 to 24 hours. No permanent sequelae were observed.

Cerebral oxygen uptake. Table IV presents the metabolic changes which followed acetazolamide injection in controls, and Table V presents these changes in subjects with hepatic encephalopathy. Figure 3 compares the CMR_{O_2} responses between the two groups. Every subject, both control and cirrhotic, exhibited an arterial pH fall after acetazolamide. Five hundred mg, or 1 g dosages evoked no blood pressure response, but when 2 g acetazolamide was administered, both control and cirrhotic subjects consistently developed a blood pressure rise (mean 20 mm Hg). Every patient, whether a control or cirrhotic, increased his cerebral blood flow considerably when

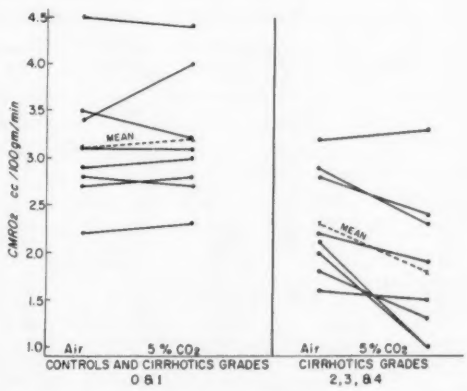

FIG. 2. CEREBRAL OXYGEN UPTAKE IN LIVER DISEASE PATIENTS AT REST (BREATHING AIR) AND DURING 5 PER CENT CARBON DIOXIDE INHALATION.

[1] One patient (M.S., with hepatitis) was examined clinically only and is not included in the metabolic data.

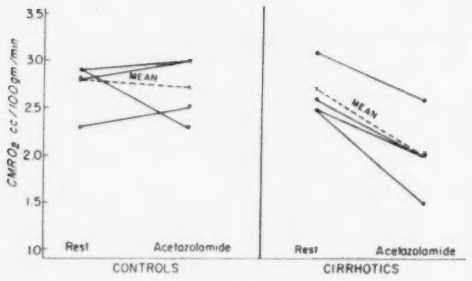

FIG. 3. CEREBRAL OXYGEN CONSUMPTION AT REST AND FOLLOWING 2 G ACETAZOLAMIDE, INTRAVENOUSLY, IN CONTROLS AND CIRRHOTICS WITH GRADE 2 ENCEPHALOPATHY.

given either 1 or 2 g acetazolamide. The cerebral oxygen uptake (CMR_{O_2}) of control subjects showed no significant ($p = > 0.8$) mean change following acetazolamide in either 1 or 2 g amounts. Similarly, the cerebral oxygen uptake of patients with hepatic encephalopathy was unaltered when only 0.5 or 1 g of acetazolamide was given. However, when the infused acetazolamide dosage was raised to 2 g (the dose required to raise blood pressure), every patient with hepatic encephalopathy showed a significant ($p = < 0.02 > 0.01$) CMR_{O_2} drop, the smallest decline being 0.5 cc per 100 g per minute.

Cerebral ammonia uptake. Four controls and all of the subjects with hepatic encephalopathy had arterial and venous ammonia levels measured while acetazolamide effects (Tables IV and V) were being determined. Neither the arterial blood ammonia level nor the cerebral ammonia uptake showed any changes correlating with the clinical or metabolic response to acetazolamide. Thus, arterial ammonias either decreased or stayed the same while cerebral ammonia uptake (CMR_{NH_3}) increased in some subjects and decreased in others.

DISCUSSION

Correlation of cerebral metabolism and encephalopathy. Our data are consistent with those of Kety (24) and Lassen (25), and show that, in metabolic encephalopathy, when psychological and somatic neurological abnormalities develop, they are accompanied by measurably impaired cerebral metabolism. Although there was some overlap between individual cases, the clinical estimate of the grade of encephalopathy and the mean CMR_{O_2} reduction correlated directly and quantitatively. Thus, cirrhotic patients graded 0 or 1 (no clinical encephalopathy) had no cerebral oxygenation depression, Grades 2 and 3 had mean oxygen uptakes of 2.9 cc and 2.0 cc per 100 g brain per minute, respectively, and the two Grade 4 (comatose) patients had brain oxygen consumptions less than 2.0 cc per 100 g per minute. The data differ from the observation of Fazekas and associates (8) that

TABLE V

Change in cerebral metabolism after acetazolamide in patients with encephalopathy

Name	Acetazolamide dose	Art. pH		Cerebral A-Vo_2		CBF		$CMRo_2$		A-VNH_3		$CMRNH_3$	
		Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.	Rest	Acet.
	g			*vol %*		*ml/100 g/min*		*cc/100 g/min*		*μg/ml*		*μg/100 ml/mm*	
Grade 4													
B.F.	1	7.52	7.51	7.38	3.68	18	33	1.3	1.2	2.40–1.64	1.80–1.76	13.7	1.3
Grade 2													
C.K.	0.5	7.52	7.51	6.63	4.86	45	57	2.9	2.8	2.87–2.56	2.94–2.74	13.9	11.4
C.K.	1.0	7.49	7.44	5.05	3.11	59	102	3.0	3.2	3.57–3.40	3.38–3.32	10.0	6.1
Grades 2–3													
F.H.	2	7.62	7.59	6.00	3.30	44	62	2.6	2.0	2.84–2.44	2.56–2.41	17.2	9.3
T.L.	2	7.51	7.47	5.20	2.07	48	73	2.5	1.5	4.12–4.12	4.05–3.90	0	10.9
H.D.	2			6.97	2.75	36	72	2.5	2.0	2.48–2.15	2.38–2.34	11.9	2.9
M.P.	2	7.47	7.46	6.19	2.54	50	103	3.1	2.6	2.86–2.69	2.91–2.68	8.5	23.4
Mean								2.7	2.0				
								$p^* = <0.02>0.01$					

* Values calculated for patients receiving 2 g acetazolamide.

mentally alert cirrhotic patients show depressed oxygen consumptions with a mean of 2.3 cc per 100 g per minute. Unless they included Group 2 patients among those classified as "alert" (26), no explanation for Fazekas' unique experience is forthcoming. Although our Group 4 sample is small, our data also fail to confirm Fazekas' finding that comatose patients showed a CMR_{O_2} no lower than still-conscious subjects.

Relationship of encephalopathy, arterial blood pH and respiratory gases. Respiratory alkalosis is one of the most consistent biochemical accompaniments of severe liver disease and few of the present subjects lacked this change, whether or not they showed neurological impairment. Vanamee and associates (1) reported that 25 out of 29 patients with hepatic encephalopathy were alkalotic, and others (4, 27) have observed similar findings. If frankly acidotic patients (those with renal failure) are omitted from the data of the Vanamee group, the mean blood pH rose progressively as encephalopathy increased. Tyor and Sieker (4), although confirming the near-ubiquity of arterial blood alkalosis, noted less correlation between the absolute pH level and the degree of encephalopathy. However, Tyor and Sieker did find in individual cases that the arterial pH rose as encephalopathy progressed. Robin, Whaley, Crump and Travis (27) noted no correlation between arterial hypocapnia and state of consciousness. Our own data correspond more with Vanamee's, since the mean arterial blood pH rose as encephalopathy progressed from Grade 0 to Grade 4. Despite the pH rise, hyperventilation was evident clinically only when neurological changes were pronounced (Grades 3, 4).

Arterial Pa_{CO_2} levels were depressed commensurately with the degree of alkalosis, being lowest among comatose subjects. Alkalosis and hypocapnia reduce cerebral blood flow (10) and impair alertness and equilibrium in the hyperventilation syndrome (28). Reduction of Pa_{CO_2} comparable with that seen in hepatic encephalopathy produces electroencephalographic (EEG) abnormalities in animals (29), indicating cerebral dysfunction. Despite the evidence that hypocapnia sometimes is deleterious, it seems doubtful that low blood carbon dioxide levels contribute importantly to hepatic encephalopathy. Hyperventilation during routine adult human EEG recordings seldom produces marked slowing unless epilepsy or hypoglycemia (30) is present. Some cirrhotic patients with intact mentation and cerebral metabolism show just as much hypocapnia as do others in coma (Table I) (1, 4). Hyperventilation alkalosis comparable with that of liver disease failed to depress cerebral metabolism in Kety and Schmidt's normal subjects (10). Finally, reversing hypocapnia with CO_2 therapy in the present study failed to ameliorate either the clinical or the metabolic manifestations of encephalopathy.

The arterial oxygen desaturation which accompanies all grades of hepatic encephalopathy (Table I) is usually modest. However, because of the respiratory alkalosis of advanced cirrhosis, the resultant Pa_{O_2} is considerably lower at any given oxygen saturation than would be the case if pH were normal (9). As a result, several cirrhotics in the present study showed alarmingly low Pa_{O_2} values. Although this depression of available oxygen may be an unfavorable influence in hepatic encephalopathy, it is an unlikely cause for the condition, since no correlation was observed by either ourselves or others (4) between mean Pa_{O_2} and depth of coma. The low values do suggest that oxygen therapy might be a useful adjunct to treating seriously ill patients with liver disease.

The mechanism of alkalosis in liver disease. The alkalosis of severe liver disease is clearly due to relative overbreathing, although the hyperventilation usually remains moderate and clinically unapparent until severe encephalopathy develops. The moderate anoxemia which often accompanies cirrhosis (4, 31, 32) is insufficient to account for the hyperpnea (33). Peripheral reflexes from the lungs stimulating the respiration (34) have not been examined. Lacking evidence for other mechanisms, most workers who have studied the problem (11, 32, 35) speculate that the hyperventilation of cirrhosis arises centrally, presumably from biochemical stimulation of respiratory center neurons.

Examples of central hyperventilation in other diseases exist, although the respiratory changes are rare in the absence of other manifestations of neurological impairment. Thus, when hyperpnea develops in metabolic acidosis, consciousness is usu-

ally impaired. Similarly, the hyperventilation which is seen with medial pontine tegmental lesions in man is accompanied by profound neurological dysfunction in nonrespiratory spheres (36). The clinical picture of severe metabolic encephalopathy with coma, muscular rigidity, fixed extraocular movements and sustained evident hyperpnea closely resembles the signs and symptoms which follow medial pontine infarction. However, hyperpnea is seen in patients with liver cirrhosis long before any other sign of presumed neurological dysfunction. This suggests that either brain stem respiratory-controlling neurons have an extraordinary susceptibility to the metabolic dysfunction of liver disease or that peripheral stimulating mechanisms need closer study than they have heretofore received.

If hyperventilation in liver disease does have a central origin, the biochemical process that either stimulates facilitatory neurons or blocks normally inhibitory respiratory nerve cells is presently unknown. Roberts, Thompson, Poppell and Vanamee (11), Sherlock (35) and others have considered raised blood ammonia to cause hyperventilation, presumably by directly stimulating the respiratory centers. Evidence to support this supposition is conflicting. There are reports that hyperventilation follows ammonia infusions in dogs (11), mice (14) and men (37). However, another carefuly controlled and detailed human experiment (38) showed no significant arterial blood pH change during ammonia infusions that produced arterial blood ammonia levels exceeding 500 μg per 100 ml. Ammonium salts infused directly into dogs' carotid arteries produced only slight and transient arterial blood pH elevations (39). These observations plus the lack of correlation between arterial blood ammonia, and either pH (4) or minute ventilation (32) reduce the likelihood that ammonia, per se, contributes significantly to hyperventilation in severe liver disease.

Another mechanism to produce central hyperventilation would be through presently unknown acid metabolites accumulating intracellularly in the brain stem respiratory centers. Advanced liver disease probably disrupts the Krebs cycle, since increased levels of pyruvate and glutamate have been found in the cerebrospinal fluid (40, 41). Hyperventilation and extracellular alkalosis would be compensatory and protective if caused by intracellular acidity of central respiratory neurons. Unfortunately, no present methods exist to explore this speculation in man.

The toxicity of carbon dioxide. Carbon dioxide inhalation clearly harmed cirrhotic patients with encephalopathy, producing both clinical deterioration and a decreased CMR_{O_2}. The deterioration could not be attributed to rising ammonia levels since both arterial ammonia levels and cerebral ammonia uptakes fell during CO_2 breathing in all instances presented here. (More extensive data are presented elsewhere indicating that in normal subjects as well as in cirrhotics, respiratory alkalosis raises blood ammonia while CO_2 breathing lowers it (42). Moreover, reducing the blood pH decreases the partial pressure of gaseous, diffusible ammonia (14, 43), and this alone should lessen the brain uptake.) Also, the deterioration could not be attributed to deleterious effects resulting from the muscular efforts of CO_2-induced hyperpnea, since similar deterioration without hyperpnea followed increasing tissue CO_2 tensions with acetazolamide.

Carbon dioxide is a toxic gas, although concentrations higher than those employed here usually are required for toxic effect. Inhaling 10 per cent CO_2 can make normal persons comatose (44). Since the subjects with hepatic encephalopathy had a low resting Pa_{CO_2}, breathing 5 per cent carbon dioxide produced a proportionately greater increase in blood and tissue CO_2 tensions than would have been produced in normals. However, several Grade 0 and Grade 1 subjects also had low resting Pa_{CO_2}'s, yet suffered no ill effects during CO_2 inhalation. Thus, the toxic responses to 5 per cent carbon dioxide could not be explained merely by different proportionate increases resulting from chronic hypocapnia.

Pre-existing cerebral metabolic derangements enhance the brain's susceptibility to many pharmacological depressants given in ordinarily innocuous dosages. Patients with structural brain disease frequently react unfavorably to modest doses of barbiturates, alcohol or anticonvulsants. This phenomenon may partially explain CO_2 toxicity in hepatic encephalopathy and also could explain why ammonia is sometimes toxic for patients with

liver disease although equivalent levels are harmless for normal subjects (38).

How carbon dioxide specificially affects nerve cell metabolism is unknown, although the gas alters the function of central neurons extending far beyond the respiratory centers (29). If the speculation that accumulating intracellular acid metabolites cause hepatic encephalopathy proved to be true, this would readily explain why carbon dioxide is toxic. The diffusibility of CO_2 across membranes rapidly makes the cerebral spinal fluid (45) and, presumably, brain cells more acidic. Other acid radicals have no such prompt effect (46). Thus, carbon dioxide would particularly intensify the already existing biochemical disturbance. In any case, there is little present evidence that respiratory alkalosis harms cirrhotics, and it is apparent that using carbon dioxide to reverse the alkalosis increases the encephalopathy.

The mechanism of acetazolamide toxicity. Clinical and cerebral metabolic deterioration was prompt and striking in the patients with hepatic encephalopathy who received 2 g of intravenous acetazolamide. Indeed, the response was such that we felt unjustified in extending the study to a larger group of cirrhotics.

Acetazolamide is not an inherently toxic drug. Normal subjects sometimes develop drowsiness and paresthesias after receiving doses of the drug exceeding 1 g (47–49), and such a response was observed to begin about 2 hours after infusion in one of our controls. This response, whose mechanism is unknown, was qualitatively entirely different from the increased confusion and mental obtundity found in the cirrhotics, and the time delay was also dissimilar to the cirrhotics' immediate worsening.

The toxic effects of acetazolamide in hepatic encephalopathy described here appear to be best explained as resulting directly from increased cerebral CO_2 tensions. Several things suggest this conclusion.

1. Acetazolamide's only known direct metabolic effect is carbonic anhydrase inhibition (50), which increases venous and tissue P_{CO_2} (51).

2. Significant CMR_{O_2} reduction in hepatic encephalopathy followed 2 g doses of acetazolamide but not lesser doses. The Henderson-Hasselbalch equation is not applicable to calculate blood gas tensions when carbonic anhydrase is inhibited (52), but two observations in this study suggested that 2 g of infused acetazolamide produced more CO_2 retention than did 1 g of the drug. Thus, the mean blood pressure rose only in subjects receiving the 2 g dose (53), and the subjects receiving 2 g showed a mean CBF rise of 87 per cent while subjects receiving 1 g or less of acetazolamide showed a mean CBF rise of but 62 per cent.

3. No consistent arterial ammonia rise accompanied the immediate clinical and cerebral metabolic deterioration which followed acetazolamide. As a secondary result of acetazolamide's inhibiting renal tubular carbonic anhydrase, urine ammonia falls and renal vein ammonia rises (54, 55). Consequently, after a period of time, the arterial ammonia rises in those cirhotics who have a decreased hepatic ammonia uptake. Owen, Flanagan, Berry and Tyor (56) found this rise 45 minutes after acetazolamide injection, and Webster and Kleinerman (57) noted a 24 per cent arterial ammonia increase 1 hour after injecting acetazolamide. Our data, which show no blood ammonia rise 30 minutes after acetazolamide, indicate that more than 30 minutes is required for impaired renal output to increase arterial ammonia.

Previously, the secondary ammonia rise described above has been regarded as the likely mechanism for acetazolamide's toxicity in patients with hepatic cirrhosis (58). The observations presented here suggest that acetazolamide's major toxic effect may be more direct and due to the increased cerebral CO_2 tensions resulting from carbonic anhydrase inhibition.

SUMMARY

1. Arterial blood gases, cerebral blood flows and cerebral oxygen uptakes were determined in control subjects, alert cirrhotic patients and patients with varying degrees of hepatic encephalopathy. The clinical and cerebral metabolic responses to inhaled 5 per cent carbon dioxide and to infused acetazolamide were tested in both cirrhotics and controls.

2. Alert cirrhotic patients had respiratory alkalosis and the degree of alkalosis increased progressively as encephalopathy appeared and worsened.

3. The cerebral oxygen uptake was normal in alert cirrhotics, but the uptake progressively de-

creased as hepatic encephalopathy developed and worsened.

4. Five per cent carbon dioxide produced no adverse effects on alert cirrhotics or patients with static brain disease. However, inhaling 5 per cent CO_2 caused both clinical deterioration and significantly decreased cerebral oxygen uptake in patients having hepatic encephalopathy. The arterial blood ammonia decreased and the cerebral ammonia uptake declined during CO_2 inhalation.

5. Acetazolamide infusions produced no significant clinical or cerebral metabolic rate (CMR) changes in control subjects. Patients with hepatic encephalopathy showed no CMR_{O_2} change when given 0.5 or 1 g of the drug. However, when patients with hepatic encephalopathy received 2 g of acetazolamide, they promptly deteriorated clinically and significantly decreased their cerebral oxygen consumption. The arterial ammonia level did not increase in any instance.

6. This study suggests that the toxicity of acetazolamide in hepatic encephalopathy directly results from increased cerebral tissue carbon dioxide tensions. It is postulated that respiratory alkalosis in advanced liver disease is compensatory, acting to protect rather than to impair brain function.

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CHARACTERIZATION OF ANTI-THYROGLOBULIN FACTORS IN HUMAN SERUM

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Investigations in patients with thyroid disease led to the discovery of serum factors which react like antibodies with thyroglobulin-containing extracts of human thyroid gland (1, 2). These serum factors have been shown to be among the γ-globulins (3). Pressman and colleagues (4) reported that the serum anti-thyroglobulin activity in one patient was associated with the γ-macroglobulins. Korngold, Van Leeuwen and Brener (5), utilizing gel-diffusion immunochemical techniques, recently reported an additional instance of anti-thyroglobulin activity associated with a γ-macroglobulin but found the activity in three other patients to be associated with the smaller 6.6S γ-globulins. Roitt, Campbell and Doniach (3), using gel-diffusion techniques to characterize the thyroid antigens, obtained data that can be interpreted as showing that the antibodies in the sera tested were the smaller, probably 6.6S, γ-globulins.

Recent studies have emphasized that γ-globulins should be regarded as an electrophoretic grouping of related but not identical proteins. They may be subdivided on the basis of size, hexose content and immunochemical properties into two major groups, the 6.6S and 18S γ-globulins, with molecular weights of about 160,000 and 1,000,000, respectively (6–8). Many physiologic and pathologic antibody activities belong in one or the other or, in some instances, in both of these γ-globulin fractions (9–11). In the present studies anion-exchange cellulose chromatography (12), which has proved to be particularly useful in γ-globulin subfractionation, was combined with electrophoretic and ultracentrifugal procedures to characterize the anti-thyroglobulin factors found in seven sera. In five patients the anti-thyroglobulin reactivity was found associated with 6.6S γ-globulins and in two cases, with both 18S and 6.6S γ-globulins.

METHODS

Anti-thyroglobulin activity in serum and serum fractions was determined by a modification of the tanned cell hemagglutination technique of Boyden (13) and Witebsky and colleagues (2). Thyroglobulin,[1] obtained from human colloid goiter and found to be 86 per cent pure by ultracentrifugal analysis, was fixed to tanned red cells. The preparation of tanned cells, procedures for dilution of serum and serum fractions, and the technique of evaluating agglutination were essentially the same as those described elsewhere (14). The specificity of the tanned-cell hemagglutinin test for thyroglobulin antibodies was tested by absorption of reactive sera with a thyroglobulin preparation which was 96 per cent pure on ultracentrifugal analysis. All of the activity was removed by this preparation, providing support for the assumption that agglutination of the red cell preparation was due to anti-thyroglobulin activity.

Analytic paper electrophoresis, quantitative total serum protein measurements, and preparative electrophoretic procedures utilizing polyvinyl particle blocks have been described (8). Ultracentrifugal fractionation was carried out in the manner of Robbins, Petermann and Rall (15) utilizing a Spinco model L preparative ultracentrifuge. Sera were diluted 1:10 with 0.15 M NaCl solution and serum fractions were dialyzed thoroughly against the same solution prior to ultracentrifugation in the Spinco no. 40 rotor at 39,000 rpm for 150 minutes. At the end of this time successive samples were collected from the top to the bottom of the ultracentrifuge tube by using a sampling syringe and needle. Determinations were made of anti-thyroglobulin activity and the results plotted in terms of the cumulative activity with increasing depth in the tube.

Anion-exchange cellulose chromatography was performed on columns of diethylaminoethyl-cellulose (16), employing 2 g of adsorbent and 1 ml of serum. The starting and equilibrating potassium buffer was 0.02 M phosphate, pH 8. A 150 ml gradient elution system providing a progressive rise in phosphate molarity to 0.25 M while maintaining pH 8, was used to elute the absorbed proteins from the column (8). Effluent from the column was collected in about 45 fractions and the protein content

[1] The authors wish to thank Dr. J. Robbins for supplying the human thyroglobulin preparations used in these studies.

estimated by measuring the optical density at 280 mμ in a Beckman spectrophotometer. The effluent was then combined into 10 or more pools which were tested for anti-thyroglobulin activity after dialysis against 0.15 M NaCl and without concentration.

RESULTS

Serological studies. Anti-thyroglobulin activity was tested in the serum of 118 patients with a variety of diseases (Table I), and 17 were found to have anti-thyroglobulin activity. Sera from 10 of the 11 patients with chronic thyroiditis had detectable anti-thyroglobulin activity. Although the diagnostic subdivision in Table I was made on clinical grounds, for it was not possible for us to obtain histologic confirmation of the diagnosis in each patient, the results showing the highest titers in chronic thyroiditis are consistent with the experience of Roitt and Doniach (17).

The observation of significant serum anti-thyroglobulin titers in four other diseases was of particular interest. One of these was a patient with Addison's disease and an enlarged thyroid gland, but no evidence of thyroid dysfunction. Patient H.L. had lupus erythematosus with no overt evidence of thyroid disease. Patient M.D., with a combination of purpura, Sjögren's syndrome and hyperglobulinemia and elevated serum macroglobulins (35 per cent), had a benign adenoma of the thyroid removed 10 years earlier. Patient E.B. had rheumatoid arthritis with evidence of thyroid dysfunction described elsewhere (18). Of interest is the finding that, in the lupus erythematosus sera and rheumatoid arthritis sera studied, only one serum from each group contained anti-thyroglobulin reactivity, although many of these patients had serum globulins which reacted with other tissue components (19).

TABLE I

Serum anti-thyroglobulin activity in disease

Clinical diagnosis	No. of patients	Hemagglutination titer (reciprocal) <10	10 to 1,280	1,280 to 3,000,000
Thyroid disease				
Chronic thyroiditis	11	1	3	7
Suspected thyroiditis	9	8	1	0
Other thyroid diseases	11	9	2	0
Lupus erythematosus	15	14	0	1
Rheumatoid arthritis	21	20	0	1
Dysproteinemia*	14	13	1	0
Other diseases	37	36	1	0
Total	118	101	8	9

* Macroglobulinemia 9, multiple myeloma 2, diffuse hypergammaglobulinemia 3.

TABLE II

Sera used for characterization of anti-thyroglobulin factors

Patient	Diagnosis	Anti-thyroglobulin titer (reciprocal)	Serum γ-globulins gram %
Normal		<10	1.17(±0.20)
M.H.	Hashimoto's thyroiditis	600,000	1.89
L.J.	Hashimoto's thyroiditis	12,800	1.58
F.S.	Hashimoto's thyroiditis	16,000	2.19
H.M.	Hashimoto's thyroiditis	3,000,000	2.35
H.L.	Lupus erythematosus	6,250	3.79
E.B.	Rheumatoid arthritis	6,250	1.60
M.D.	Sjögren's syndrome*	1,280	5.50

* And other diseases; see text.

Seven sera were chosen for further study. Table II lists the patients' diagnoses, serum anti-thyroglobulin titers and γ-globulin levels. The first four patients had the type of chronic thyroiditis diagnosed as Hashimoto's disease. The remaining three (H.L., M.D., E.B.) have been noted above. Absorption of these seven sera with a preparation shown to be at least 96 per cent thyroglobulin removed all reactivity with the thyroglobulin-extract-coated red blood cells and indicated that the reactive material was probably specific for thyroglobulin.

Electrophoretic studies. In all seven sera the total γ-globulin level was greater than normal (95 per cent normal confidence limits: 0.77 to 1.57 g per 100 ml). The diffuse appearance of the γ-globulin increase is apparent in Figure 1 where zone electrophoretic fractionation of the M.H. serum is presented. Examination of the block fractions for anti-thyroglobulin reactivity revealed this activity to be associated with the γ-globulins. The γ-globulin character of the reactivity in this and the other sera was confirmed by chromatographic studies reported below.

Ultracentrifugal studies. Ultracentrifugal studies were undertaken to determine the sedimentation properties of the γ-globulins possessing anti-thyroglobulin activity. Figure 2 illustrates the findings when whole sera were sedimented in a preparative ultracentrifuge under conditions which would cause only partial sedimentation of 6.6S γ-globulins but almost complete sedimentation of 18S γ-macroglobulins. Five sera (H.L.,

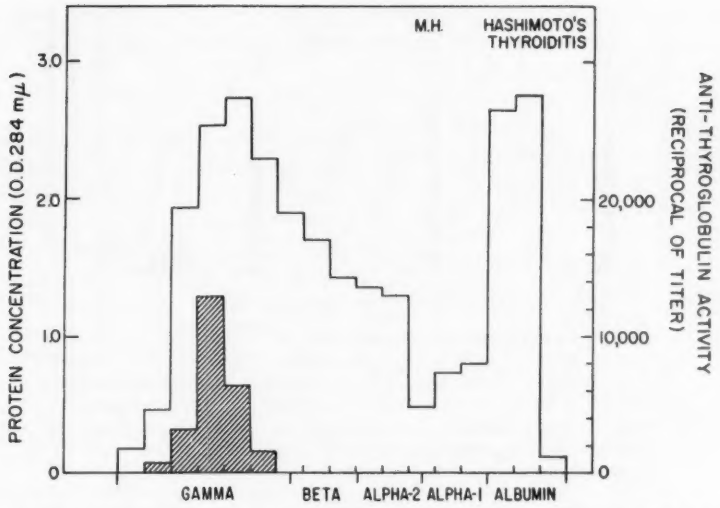

FIG. 1. DISTRIBUTION OF ANTI-THYROGLOBULIN ACTIVITY AFTER ZONE (BLOCK) ELECTROPHORETIC FRACTIONATION OF THE M.H. SERUM. The shaded area represents the distribution of anti-thyroglobulin activity. The distribution of serum protein components was roughly determined by the location of pigment bands, pink in the β region, blue in the α-2 region and pale yellow in the albumin region, and was confirmed by analytic electrophoresis of the block sections.

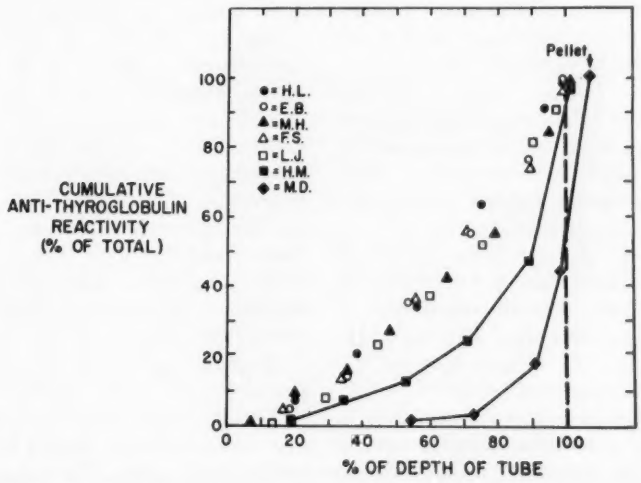

FIG. 2. SEDIMENTATION CHARACTERISTICS OF ANTI-THYROGLOBULIN ACTIVITIES IN WHOLE SERUM. Ultracentrifugation was conducted in a preparative ultracentrifuge (39,000 rpm, 150 minutes, 0.15 M NaCl). After centrifugation successive samples were removed from each tube starting at the top. The distribution of anti-thyroglobulin reactivity is expressed in terms of cumulative per cent of the total in each tube.

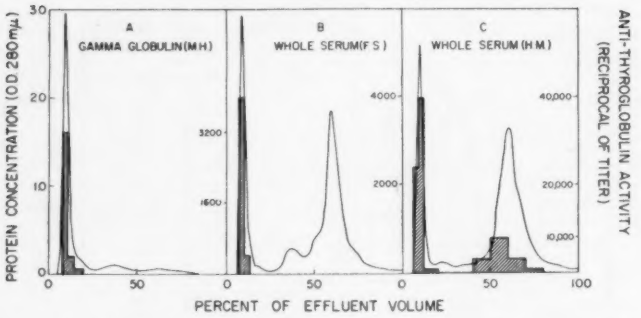

FIG. 3. CHROMATOGRAPHIC DISTRIBUTION OF ANTI-THYROGLOBULIN ACTIVITY IS SHOWN IN A, γ-GLOBULIN OBTAINED BY PREPARATIVE ELECTROPHORESIS; IN B, WHOLE SERUM WITH THE ACTIVITY IN A SINGLE CHROMATOGRAM REGION; AND IN C, WHOLE SERUM WITH ACTIVITY IN TWO CHROMATOGRAM REGIONS. Chromatography was conducted on diethylaminoethyl-cellulose columns at pH 8, and proteins were eluted by means of a gradient of increasing salt concentration, as noted in Methods.

E.B., M.H., F.S., L.J.) showed a uniform pattern of activity distribution, consistent with the sedimentation properties of 6.6S γ-globulins. In sera of H.M. and M.D., however, much of the activity sedimented more rapidly, suggesting that the anti-thyroglobulin activity was associated either with a molecule that sedimented more rapidly than the other serum factor or was associated with two types of molecules, at least one of which sedimented more rapidly.

Chromatographic studies. Whole sera and γ-globulins prepared by electrophoretic fractionation of whole sera were further fractionated by anion-exchange cellulose chromatography. Representative chromatograms together with the distribution of anti-thyroglobulin activity are presented in Figure 3. A single peak of antibody activity was found in the range of 5 to 15 per cent of the elution volume after chromatography of whole sera or γ-globulins from Patients M.H., F.S., E.E., M.H. and L.J. (Figure 3, A and B). The proteins in this peak are known to be 6.6S γ-globulins (8), and finding of activity solely in this protein region is consistent with the uniform ultracentrifugal sedimentation properties of the activities in these same sera (Figure 2). Electrophoretic, chromatographic and ultracentrifugal observations were all consistent with the association of the anti-thyroglobulin activity with 6.6S γ-globulin molecules.

The anti-thyroglobulin activities were more complex in the M.D. and H.M. sera. Anion-exchange cellulose chromatography revealed two peaks of antibody activity. The H.M. serum chromatogram is illustrated in Figure 3C. The M.D. chromatogram was qualitatively similar. Anti-thyroglobulin activity was found in the 5 to 15 per cent and also in the 50 to 80 per cent effluent region. With the H.M. serum, very low titers were obtained in the 15 to 40 per cent effluent regions (Figure 3C). The finding of activity in the 50 to 80 per cent region, which is composed primarily of 18S γ-macroglobulins, suggested that anti-thyroglobulin activity in this fraction was a property of 18S γ-globulins. The chromatographic separation of activity into two groups demonstrated that in the H.M. and M.D. sera two different antibodies possessed anti-thyroglobulin activity and distinguished these sera from the other five sera.

Ultracentrifugal studies, illustrated for the M.D. sera in Figure 4, demonstrated that the activity in the 60 to 80 per cent chromatogram effluent region sedimented more rapidly than that in the 5 to 15 per cent region. The sedimentation behavior of the anti-thyroglobulin activity in the 60 to 80 per cent chromatogram region was consistent with that of an 18S γ-globulin. These observations indicate that anti-thyroglobulin activity in these two sera were properties of both 6.6S and 18S γ-glob-

ulins and that these antibodies were separable by anion-exchange cellulose chromatography.

DISCUSSION

The present study confirms the observations that anti-thyroglobulin activity in man may be associated with 6.6S or 18S γ-globulin molecules. In five patients the activity was associated solely with 6.6S γ-globulin molecules, but in two other patients anti-thyroglobulin activity was found with 6.6S and 18S γ-globulins. Little is known of the role of antigen, host or disease factors in determining whether 18S instead of 6.6S γ-globulin antibodies are to be formed. As far as we could determine in the present study the several serum antibody activities were reacting with the same substance in our thyroglobulin test. Thyroglobulin preparations, however, are not absolutely pure, and thyroglobulin is a large molecule likely to have a variety of antigenic sites. Antibodies to other large molecular substances, such as the erythrocyte isoagglutinogens, Rh factors and certain liver nucleoproteins, have also been found in both 6.6S and 18S protein fractions (9–11, 19, 20). In each instance the potential antigen is a complex compound and the several antibodies could be directed against different antigenic sites, although this possibility has not been proved.

No clinical characteristic could be discerned which would help to distinguish between the patients with 18S serum anti-thyroglobulin activities and those with only 6.6S anti-thyroglobulin antibodies. Patient M.D. presented an unusual disease pattern, with long standing anemia, hyperglobulinemia, Sjögren's syndrome and repeated episodes of purpura, and she may have an abnormal immune mechanism manifest in multiple immune abnormalities, one being the serum anti-thyroglobulin antibody. The other patient, H.M., however, with a quite different disease pattern, not atypical of myxedema, had little in common with the former patient. It is also worth noting that the 18S macroglobulin level in the H.M. serum was normal (2.7 per cent).

That the clinical and immunological significance of 18S as distinct from 6.6S antibodies remains largely unexplored has been in part due to difficulties in separating and identifying the different antibody forms. Anion-exchange cellulose chromatography has greatly facilitated the separation of physicochemically different antibodies in other antibody studies (9–11) as well as in the present

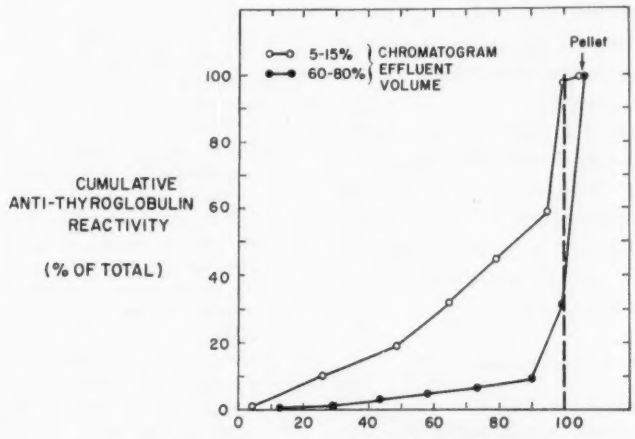

FIG. 4. DIFFERENCES IN THE SEDIMENTATION CHARACTERISTICS OF TWO ANTI-THYROGLOBULIN COMPONENTS (5 TO 15 AND 60 TO 80 PER CENT) OBTAINED BY CHROMATOGRAPHIC FRACTIONATION OF SERUM OF M.D. Ultracentrifugation was carried out as in Figure 2.

study of serum anti-thyroglobulin activity. Further application of these procedures may be expected to provide new insight into the nature of antibodies in thyroid and other diseases.

SUMMARY

Sera from 118 patients with a variety of diseases were examined for anti-thyroglobulin activity by the agglutination of red blood cells treated with tannic acid and coated with human thyroglobulin. Anti-thyroglobulin activity was found most commonly but not exclusively in patients with clinical evidence of chronic thyroiditis. The serum factors responsible for anti-thyroglobulin activity were characterized in detail in seven sera, including four from patients with chronic thyroiditis.

Anti-thyroglobulin activity was found only in the γ-globulin fraction of the serum proteins. The activity was found to be a single component with the sedimentation characteristics of 6.6S γ-globulins in five sera. In two sera, however, anti-thyroglobulin activity was found in two components with the sedimentation characteristics of 6.6S and 18S γ-globulins, respectively.

The 6.6S and 18S types of anti-thyroglobulin activity could be separated and their recognition facilitated by anion-exchange cellulose chromatography. Chromatographic separation, in addition, made possible quantitative measurement of each of the two types of activity.

The possible role of antigen and host factors in determining the type of antibody response was briefly considered. No common clinical feature in the two patients producing 18S anti-thyroglobulin antibodies could be found which would distinguish these two patients from the 6.6S antibody-producing patients.

ACKNOWLEDGMENTS

We wish to thank Drs. J. E. Rall, K. J. Block and J. Nassou for making available sera utilized in these studies. We also are indebted to Mr. Robert Bowser for help in the anti-thyroglobulin assays and ultracentrifugal procedures, Miss Ann P. Horbett for zone electrophoretic fractionations and Miss Mary E. Lawrence for assistance with the chromatography.

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FEVER FROM PATHOGENIC FUNGI *

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Most studies of fever have dealt with the pyrogenic activity of the bacterial lipopolysaccharides of gram-negative bacteria (1). The pyrogenic properties of pathogenic gram-positive bacteria, on the other hand, have been the subject of only a few reports. It is of interest, however, that intravenous injection of living staphylococci and corynebacteria were found to produce higher fever in rabbits than did gram-negative bacteria (2), and that pronounced elevations of body temperature were found after intravenous injections of both living and heat-killed streptococci, as well as streptococcal lysates (3). The pyrogenicity of another group of gram-positive microorganisms, the pathogenic fungi, has received no consideration in the literature, although Salvin (4) reported that they possess lethal endotoxins for mice. The following study was undertaken, therefore, to determine which of the common pathogenic fungi can induce fever and to compare their pyrogenic effect with that of the lipopolysaccharides of gram-negative bacteria. The presence of pyrogens in saprophytic molds has been reported but the characteristics of the pyrogen and of the fever response were not described (5).

METHODS AND MATERIALS

Source of pathogenic fungi. All but one strain were isolated from human infections. The strain of *Candida albicans* was cultured from the blood of a patient with fatal hematogenous candidiasis (6), and the strain of *Cryptococcus neoformans* from the spinal fluid of a patient with fatal cryptococcal meningitis. Two strains of *Histoplasma capsulatum* were obtained from Dr. Chester Emmons, of the National Institutes of Health. One of these (*H. capsulatum* G-3996) was isolated from soil and the other (H-635) at autopsy. The Hattie Davis and Sautman strains of *Blastomyces dermatitidis* were provided by Dr. Robert Abernathy and *Sporotrichum schenckii*, strain 7019, was obtained from the American Type Culture Collection.

Suspensions of fungi. Fungi were washed from the surface of their culture medium with a pyrogen-free buffer (pH 7.1) containing NaH_2PO_4 1.6 g, Na_2HPO_4 7.5 g, and NaCl 4.4 g per L H_2O, washed 3 times by centrifugation and suspended in 1.0 ml of the buffered saline to give the desired number of fungus cells. The fungus cells were enumerated in a Spencer bright line Neubauer hemocytometer counting chamber after dilution in saline in a red cell or white cell pipet.

Pyrogen studies. Living or autoclaved fungus suspensions were injected into the marginal ear vein of rabbits and temperatures were measured rectally by inserting the glass probe of the thermistor apparatus (Sargent). Autoclaving of fungi was done at 145° C for 15 minutes at 15 pounds pressure, and always sterilized the culture. Glassware and needles were rendered free of pyrogens by heating in an oven at 170° C for 2 hours. Pyrogen-free solutions of physiologic saline and water were purchased from the Abbott Company. Albino rabbits of both sexes, weighing 3 to 5 kg, were kept in an air-conditioned room at approximately 70° F. They were conditioned by measuring the temperatures hourly for 8 hours on the day preceding each experiment and animals were used only if their temperatures remained within a constant range of plus or minus 0.3° F during the period of conditioning. On the day of the experiment, temperatures were measured at 30-minute intervals for 2 hours before injection and hourly for 8 hours thereafter. Temperatures were also taken immediately before injection and 30 minutes afterward. Animals were rejected for the study if their temperatures were not constant during the preliminary period of 2 hours before injection.

EXPERIMENTAL RESULTS

A. Fever

1. Cryptococcus neoformans

The entire growth obtained after culture for 11 days at room temperature on the surface of Sabouraud-dextrose agar was suspended in buffered saline. One ml of a heavy turbid suspension of living cryptococci, containing about 2.5×10^8 yeast cells, was then injected intravenously into three rabbits. No fever resulted on the day of injection or on three subsequent days of observation. The rabbits showed no signs of illness from the injection. The experiment was repeated with a 71 day old culture of *C. neoformans*. An inocu-

* Supported by a grant from the American Trudeau Society.

lum of 2.5×10^8 cells again failed to produce fever.

An attempt was made to release a pyrogen by autoclaving and agitating with glass beads the suspension of 10^9 cryptococci per ml until they appeared fragmented, had lost their gram-staining properties, and stained only faintly with safranine. The disrupted cryptococci were then centrifuged at 2,000 rpm for 4.5 hours and 5.0 ml of supernate was injected intravenously into each of five rabbits. Three rabbits developed fever with peaks of 1.2°, 2.4°, and 4.0° F, respectively. Fever began to rise after latent periods of 30 to 60 minutes and the temperatures returned to normal 8 to 10 hours after injection. Two rabbits did not develop fever.

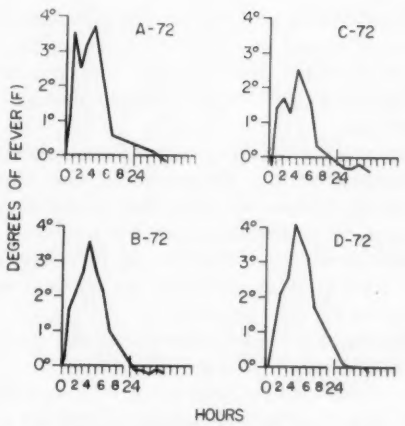

FIG. 2. FEVER RESPONSE FROM 1×10^9 AUTOCLAVED *CANDIDA ALBICANS* (SUHR) INJECTED INTRAVENOUSLY.

2. Candida albicans

a. Living agar cultures. Intravenous injection of 1 ml of a suspension of a five day culture of *C. albicans* grown on Sabouraud-dextrose agar at 27° C and containing 10^9 yeast cells produced high fever in four rabbits. After a latent period with no fever, lasting 30 minutes, the temperature rose 0.8° to 2.8° F at 1 hour and reached a peak of 3.8° to 5° F at 4 hours. The temperatures then fell to 1.6° to 2° F at 7 hours but rose again to similar peaks by 24 hours. The fever remained elevated until all animals died before 48 hours (Figure 1, C-71).

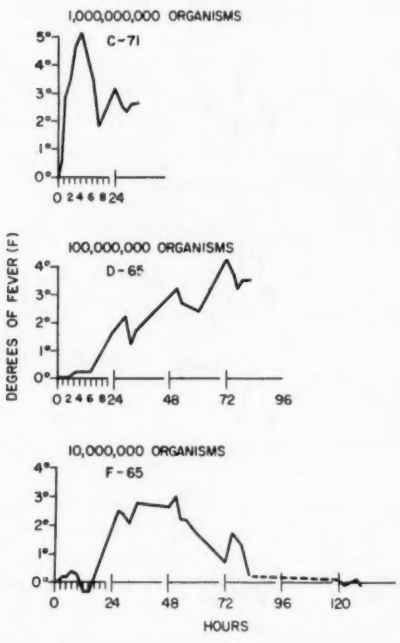

FIG. 1. FEVER RESPONSE TO LIVE *CANDIDA ALBICANS* INJECTED INTRAVENOUSLY.

b. Living broth cultures. The growth at 27° C in a four day culture in trypticase soy broth was collected on the filter pad during Seitz filtration and washed three times in buffered saline. The organisms were then suspended in the buffered saline to produce a count of approximately 10^9 yeast cells per ml and tenfold serial dilutions were made to give suspensions with 10^8 and 10^7 cells per ml, respectively. One ml of each serial dilution was then injected intravenously. The rabbits given 10^9 organisms collapsed at 3 hours, developed extreme hypothermia to a level 4° F below the initial temperature and died. Two rabbits inoculated with 10^8 candida cells remained afebrile for 2 hours and then developed a rise in temperature of 1.2° and 2.0° F at 3 hours. The temperature in both animals continued to rise to 1.8° and 2.8° F at 5 hours and remained elevated during the four day period of observation. A third rabbit inoculated with 10^8 organisms remained afebrile the first day but developed a high fever at 2.3° F the second day and this continued to mount during the four day period.

Inoculation of 10^7 living candida cells into three rabbits produced essentially no fever on Day 1, but all three had fevers on Day 2 ranging from 0.8 to 2.4° F. The fever continued through the fourth day.

One to 2.0 ml of blood collected during periods of highest fever from the central ear veins of each of the six rabbits, 48 hours after injection, was cultured on Sabouraud's agar, but no growth of candida developed. The dose of 10^8 yeast cells was lethal in about one week, but all three rabbits given 10^7 cells survived.

Representative fever curves for each dose of viable candida are shown in Figure 1.

c. Autoclaved agar culture. A 48 hour culture of *C. albicans* grown on Sabouraud-dextrose agar at 27° C was autoclaved, washed three times, and suspended in buffered saline in a concentration of 10^9 organisms per ml. One ml was then injected into the marginal ear vein of each of two rabbits. Another two rabbits were inoculated intravenously with 10^9 autoclaved candida cells that had been grown in trypticase soy broth at room temperature for 48 hours and washed three times in buffered saline. All rabbits developed high fever and the fever of two was biphasic (Figure 2). There was no difference in fever response to the two types of cultures. In three rabbits there was a latent period of 30 minutes before the temperature began to rise, but fevers of 0.8° to 3.4° F were noted in all four at 1 hour and maximum peaks of 2.4° to 4° F were present at 4 hours. Thereafter, the temperature fell rapidly and in two animals reached normal levels by 7 hours. No fever was noted the following day and the animals showed no ill effects from the inoculation either during the fever or afterwards.

d. Culture filtrates. A culture of *C. albicans*, incubated in trypticase soy broth at room temperature for four days, was passed through a Seitz filter and 10.0 ml of clear sterile filtrate was injected intravenously into two rabbits. Two control rabbits were injected intravenously with 10.0 ml sterile trypticase soy broth. The culture filtrate produced a brisk rise in temperature to 1.5° F in both animals at 1 and 2 hours after an afebrile lag period of 30 minutes. After 2 hours the temperature fell sharply and reached normal by

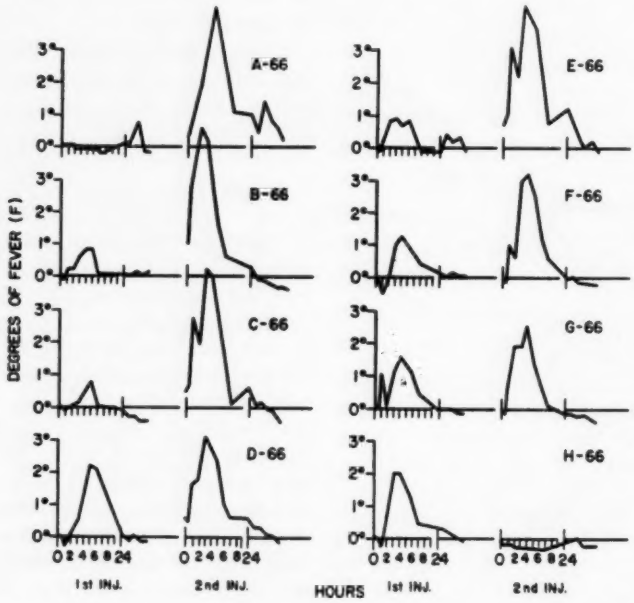

FIG. 3. FEVER RESPONSE AFTER FIRST AND SECOND INTRAVENOUS INJECTION OF LIVE *HISTOPLASMA CAPSULATUM* (AT TWO-WEEK INTERVALS).

4 hours. The animals showed no ill effects from the injection.

No fever was produced by injecting plain sterile broth in control animals.

3. Histoplasma capsulatum

a. Living agar cultures. A culture of *H. capsulatum* (G-3996), incubated at room temperature for 23 days, was removed from Sabouraud-dextrose agar with sterile buffered saline and reduced to an even suspension in a tissue homogenizer. Each of a group of eight rabbits received intravenously 1.0 ml of a saline suspension adjusted to contain 10^6 tuberculate chlamydospores per ml (Figure 3). Seven of the animals developed fever. In two animals the fever was less than 1° F; in the remainder it ranged from 1° to 2.2° F. There was a lag period of 2 hours before the temperature began to rise in five febrile animals and of 1 hour or less in two others; this lag period resembled that observed in animals given 10^8 living candida organisms. The temperature returned to normal in 6 to 8 hours and remained normal the next day. The animals showed no ill effects from the injection.

b. Autoclaved agar culture. A culture of *H. capsulatum* (H-635), grown on Sabouraud-dextrose agar for 45 days at room temperature, was suspended in saline by the method described in 3a and autoclaved. Six rabbits were then injected intravenously with 1.7×10^6 tuberculate chlamydospores in 1.0 ml of the sterile autoclaved suspension. Five rabbits experienced immediate high fevers reaching 1.5° to 4.6° F without the lag period observed in rabbits given the living culture. The temperature rise was started when the first temperature was taken 30 minutes after injection. By 7 to 8 hours the temperature was back to normal.

4. Blastomyces dermatitidis

a. Living agar culture. After growth for 29 days at room temperature a culture of *B. dermatitidis* (Davis strain) was removed from Sabouraud-dextrose agar and a suspension prepared as described in 3a. Four rabbits received intravenously 1.0 ml of a suspension of living organisms containing approximately 3.5×10^7 spores. All animals developed high fevers reaching 1.6° to 3°

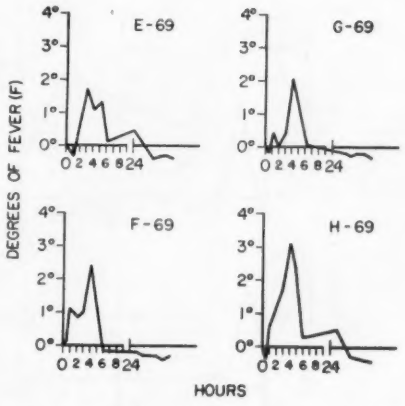

FIG. 4. FEVER RESPONSE TO INTRAVENOUS INJECTION OF 35,000,000 SPORES OF LIVE *BLASTOMYCES DERMATITIDIS.*

F (Figure 4). The temperature rose after a lag period of 30 minutes and usually reached a peak in 4 hours. Then the temperature fell abruptly to normal by 6 hours. There was no fever the next day and the animals did not appear ill.

b. Autoclaved agar culture. A culture of *B. dermatitidis* (Sautman strain) grown on Sabouraud-dextrose agar for 42 days at room temperature was suspended in saline and autoclaved. Each of five rabbits was then injected intravenously with 1.0 ml of a suspension containing 2.5×10^7 spores. Four of the rabbits developed fevers ranging from 0.6° to 3.6° F (Figure 5, first injection). In each case there was a preliminary drop in temperature at 30 minutes followed by fever peaks at 3 to 4 hours. The injections were well tolerated.

5. Sporotrichum schenckii

A yeast phase culture of *S. schenckii,* grown on trypticase soy agar with 10 per cent rabbit blood at 37° C for six days, was removed in sterile buffered saline, washed three times by centrifugation, and resuspended so that there were 1.75×10^9 organisms per ml. One ml was then injected intravenously into each of four rabbits. All developed high fever reaching 2.0° to 4.6° F (Figure 6). The lag phase varied from 30 to 60 minutes and the peak was reached at 5 hours in all animals. No ill effects were apparent from the injection.

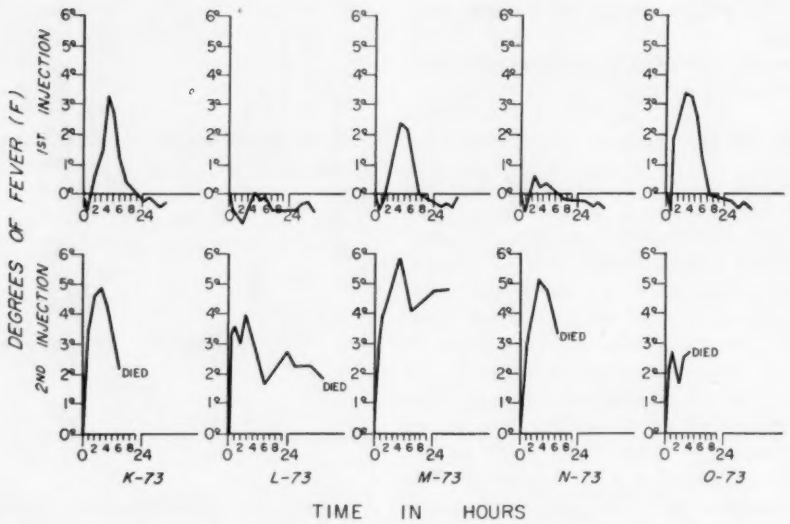

FIG. 5. FEVER RESPONSE AFTER FIRST AND SECOND INTRAVENOUS INJECTION OF 2.5×10^7 AUTOCLAVED *BLASTOMYCES DERMATITIDIS* (AT TWO-WEEK INTERVALS).

Two of the four rabbits developed fever after the first day. One animal showed daily spikes for eight days and then the temperature remained normal. Another sustained a high spike, 4.4° F, on the second day but not afterwards.

B. Hematologic Changes Accompanying Fever

Total and differential leukocyte counts were made on the blood obtained from the ear veins of the four rabbits given 10^9 living *C. albicans* and in the four given 10^9 autoclaved organisms. All

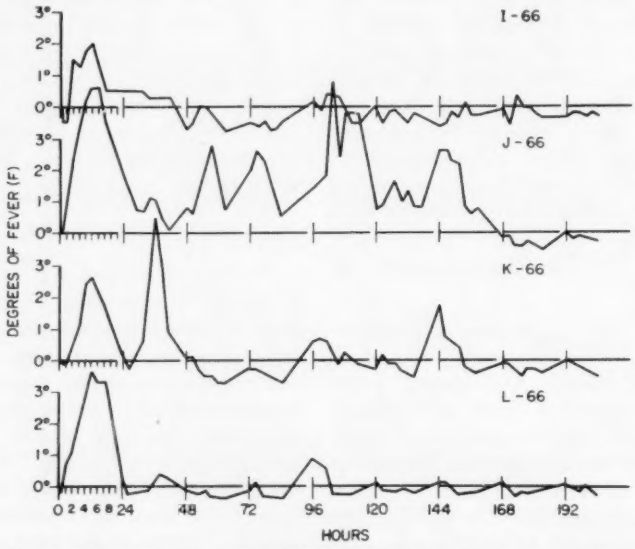

FIG. 6. FEVER RESPONSE TO 1.75×10^9 *SPOROTRICHUM SCHENCKII* INJECTED INTRAVENOUSLY.

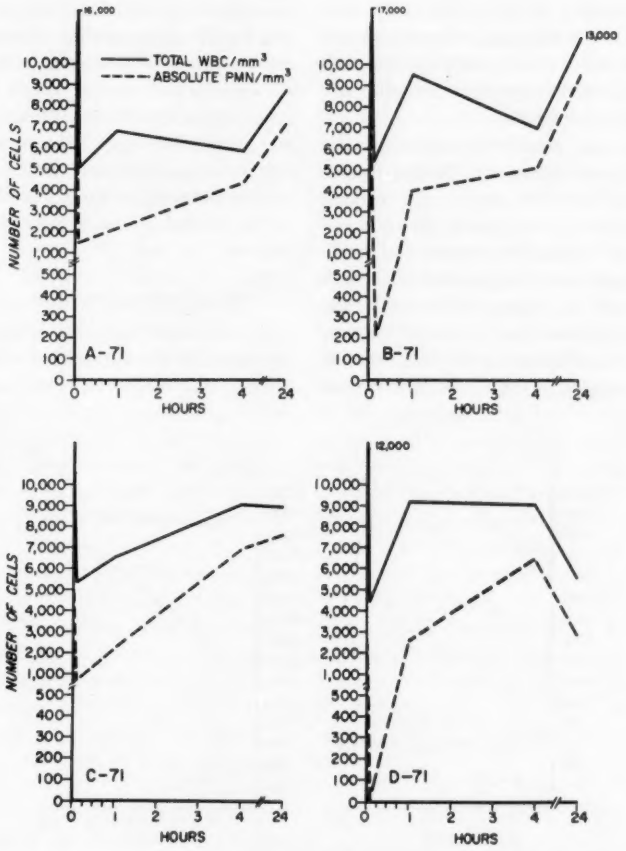

FIG. 7. BLOOD LEUKOCYTE RESPONSE TO INTRAVENOUS INJECTION OF 10^9 VIABLE *CANDIDA ALBICANS*.

leukocyte counts were made in duplicate before injection of yeast and at intervals of 5 minutes, 1 hour, 4 hours and 24 hours after injection. As shown in Figures 7 and 8, both living and dead candida produced a sharp neutropenia within 5 minutes after injection and a rebound to normal or above in 1 to 4 hours.

C. Effects of Repeated Injections on Fever

1. Candida albicans

Two rabbits that had received 5×10^8 autoclaved candida intravenously 24 days earlier were given second intravenous injections with 5×10^8 autoclaved candida. This produced higher and more prolonged fever than that experienced with the initial injection 24 days previously, but no fever was present the following day. Two weeks later a series of five daily injections of the same inoculum was given but no consistent evidence of a gradually diminishing febrile response (tolerance) was found. The animals became weaker and more listless with each injection.

Four additional rabbits, that had received 4.5×10^9 autoclaved *C. albicans* intravenously 14 days earlier, were given second intravenous injections of the same dose. Three of these sustained higher and more prolonged fever than that elicited by the first injection two weeks previously. Daily injections of the same inoculum, given for a

total of five consecutive days, produced no definite evidence of a diminishing response except in one rabbit, and this animal reacted on the fifth day with a fever response greater than that observed with the first injection.

The possibility was considered that tolerance was not demonstrated because the dose of fungus pyrogen may have been too large. An attempt was made, therefore, to determine the minimal pyrogenic dose and repeat the attempts to establish tolerance. Autoclaved suspensions of *C. albicans* were prepared in pyrogen-free saline and serial tenfold dilutions were made so that the final concentrations of fungus cells were 10^6, 10^7 and 10^8 per ml, respectively. A total of nine rabbits was then examined for tolerance by inoculating 1 ml of each concentration intravenously into three rabbits at daily intervals. The lowest dose of 10^6 candida cells proved to be a minimal pyrogenic dose and produced fevers reaching 0.5° to 0.8° F and lasting 4 hours. No sign of a diminished pyrogenic response was observed in any of the nine rabbits after eight daily injections. Instead, the fevers tended to be greater with successive injections.

2. Histoplasma capsulatum

a. Live agar culture. Eight rabbits that had received 10^6 live tuberculate chlamydospores (see Section 3a) were each challenged again intrave-

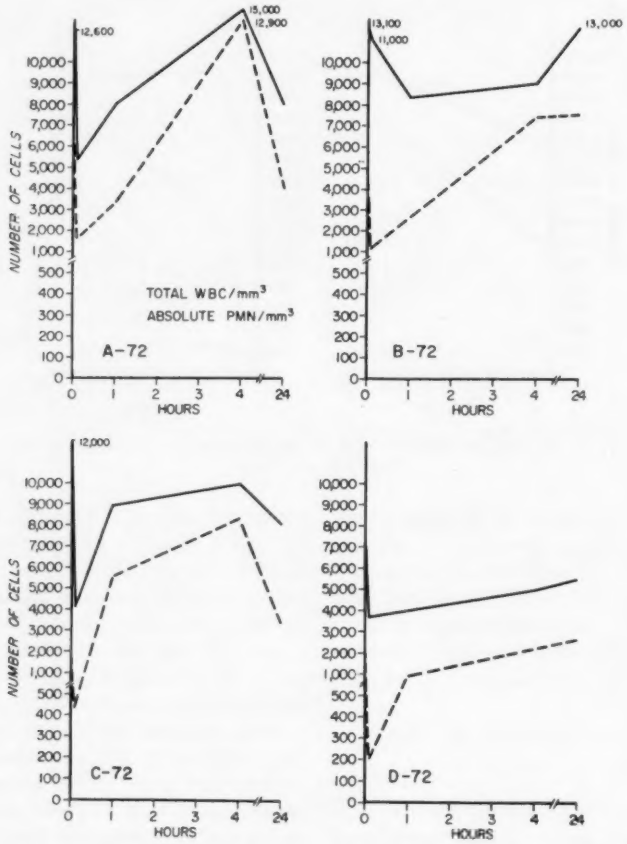

FIG. 8. BLOOD LEUKOCYTE RESPONSE TO INTRAVENOUS INJECTION OF 10^9 AUTOCLAVED *CANDIDA ALBICANS*.

nously two weeks later with 10^6 live histoplasma grown for 18 days on Sabouraud-dextrose agar at room temperature. The fever in seven of the rabbits was much higher and prolonged than at the time of the first injection, when the same number of chlamydospores was inoculated. In all seven rabbits the fever rise was more than 2°, in five, more than 3° and in four, more than 4° F. At the time of the first injection, on the other hand, the temperature of only one animal rose above 2° F (2.2). The second curves also tended more than the first to be biphasic (Figure 3).

b. Autoclaved agar culture. Five rabbits given the autoclaved agar culture of *H. capsulatum* described in 3b were given a second intravenous injection of the same inoculum containing 1.7×10^6 tuberculate chlamydospores on the next day. Three died within a few hours after the injection and the remaining two were found dead the next morning. Only one animal exhibited a significant fever.

In order to determine the role of hypersensitivity in the rapid deaths that followed the second injection of *H. capsulatum,* skin tests were performed with histoplasma filtrates. The autoclaved fungus suspension used for intravenous injection was centrifuged at 2,000 rpm and 0.1 ml of the clear supernate was injected intradermally in the mid-portion of the back of three rabbits 24 hours after each had received 1.7×10^6 chlamydospores intravenously. An intradermal injection of 0.1 ml normal saline served as a control in the same rabbits. Identical tests with histoplasma filtrate and saline were performed in three control rabbits that had received no intravenous injection of fungi. No dermal hypersensitivity was found in the rabbits inoculated intravenously with the histoplasma cells 24 hours before skin testing. The reaction produced by injecting the histoplasma filtrate was the same in test animals and controls. During the first hour the swelling at the test site was no greater than that at the saline site; and at 24 hours slight erythema persisted at the test sites in both groups of animals.

3. Blastomyces dermatitidis

Five rabbits were given a second intravenous inoculation of 2.5×10^7 spores of autoclaved *B. dermatitidis* (Sautman strain) 14 days after the first. The comparative febrile responses to the two injections are shown in Figure 5. Four of the animals sustained fevers higher than 4.0°, and three, higher than 5.0° F. In all animals the lag period was abolished and high fevers were present at 30 minutes. Unlike the first injection, the second killed four animals. The outstanding changes postmortem were pulmonary hyperinflation and engorgement with blood and edema fluid.

No immediate or delayed dermal hypersensitivity to Blastomycin[1] or to the supernate from the autoclaved blastomyces suspension was noted in four additional rabbits subjected to skin tests 14 days after intravenous injection of autoclaved *B. dermatitidis.* The techniques for preparation of the supernate and for skin testing were the same as those described above for histoplasma supernates. Three normal rabbits that had never received fungus injections served as controls.

The same accelerated and increased fever response was seen in rabbits given this inoculum of autoclaved *B. dermatitidis* 14 days after the inoculation of living *B. dermatitidis* (3.5×10^7 spores of the Davis strain). Four such infected animals developed fevers of over 5° F and died. These animals also showed severe pulmonary engorgement and overdistention. In addition, there were blastomycotic abscesses in the lungs and kidneys.

4. Cryptococcal lysate

Five ml of the cryptococcal lysate prepared by disrupting the cells with glass beads (as described in A1) was injected intravenously daily into each of five rabbits. All developed fever with no evidence of a diminished response after one week of consecutive daily injections. Instead, all showed gradually increasing fever with succeeding injections of the yeast cell lysate.

D. Passive Transfer of Circulating Pyrogen

Nine rabbits were inoculated intravenously with a suspension of 5×10^8 *C. albicans* prepared as described in Section A2a. One died before 24

[1] Blastomycin was obtained from Parke, Davis and Co., Detroit. It is described as a sterile filtrate from the culture of the mycelial phase of *B. dermatitidis* grown on liquid synthetic medium. Each rabbit received 0.1 ml of Blastomycin intradermally.

hours, five were exsanguinated at 24 hours, and three were exsanguinated at 48 hours. The temperature elevations at the time of bleeding at 24 hours ranged from 1.9° to 6.2° with an average of 3.7° F; at 48 hours they ranged from 1.1° to 2.7° with an average of 1.8° F. The sera obtained at 24 hours and 48 hours were pooled separately. Three ml of the 24 hour pool and 3.0 ml of the 48 hour pool were cultured on trypticase soy agar and trypticase soy broth, and both pools proved to be sterile. The 24 hour pool was divided into five portions of 30 ml and the 48 hour pool into two 25-ml portions. Each portion of pooled serum was injected intravenously into a normal rabbit. Three rabbits receiving the 24 hour pooled serum developed fevers lasting 3 hours with single peaks in each at 1 hour of 1.0°, 1.6°, and 1.7°, respectively. No fever occurred in the two rabbits given 48 hour pooled serum. *C. albicans* was recovered by culture in enormous numbers from the kidneys of all nine donor rabbits sacrificed at 24 and 48 hours after injection. These infected kidneys were studded with microscopic mycotic abscesses which became grossly evident in other rabbits followed for 76 hours (Figure 9). The absence of a pyrogen in the 48 hour serum may have been related to the fact that the fevers were lower than at 24 hours when serum was found to contain a pyrogen.

E. Pyrogen Controls

No rise in temperature resulted from the injection of 1.0 ml of each of the following materials intravenously in rabbits: *1*) Buffered saline. *2*) Trypticase soy broth. *3*) Agar washings prepared from Sabouraud-dextrose agar by flooding it with buffered saline and scraping bits of agar loose from the surface. *4*) Agar washings prepared in a similar fashion from trypticase soy agar containing 10 per cent rabbit blood. These washings developed a strong tinge of color from the hemoglobin.

DISCUSSION

The pyrogenic properties of the pathogenic fungi examined in this study were similar in many respects to those of the gram-negative bacteria and their lipopolysaccharide pyrogen. The pyrogens of both fungi and gram-negative bacteria resist heat, pass through Seitz filters, induce neutropenia and elicit similar fever curves. These fever curves are characterized by: *1*) a lag pe-

FIG. 9. MYCOTIC ABSCESSES OF RABBIT KIDNEYS 96 HOURS AFTER INTRAVENOUS INOCULATION OF 5×10^6 VIABLE *CANDIDA ALBICANS*.

riod, *2*) one or two distinct peaks, and *3*) a total duration of 7 to 8 hours with large (but not overwhelming) doses. These similarities between pyrogenicity of the gram-positive fungi and gram-negative bacteria provide additional evidence that endotoxic properties are not limited to the latter group of microorganisms (3, 4). The only apparent difference between the fungus pyrogen and the gram-negative pyrogen was found upon repeated injections. Repeated injections of the pyrogens of gram-negative organisms lead to diminished febrile reactions and greater resistance to the other toxic effects (1). No convincing evidence of such tolerance was found upon repeated injections of fungi; instead the pyrogenicity and toxicity were usually enhanced. For example, a second injection of autoclaved histoplasma organisms 24 hours after the first was lethal. While two consecutive injections of bacterial lipopolysaccharide can also kill rabbits through the effects of the generalized Shwartzman reaction, preliminary evidence indicates that the phenomena are not related. The generalized Shwartzman reaction is not readily produced in adult rabbits and does not elicit the rapid (often instantaneous) deaths observed with a second daily dose of *Histoplasma capsulatum* (7). A similar susceptibility to injury by fungi was reported by Henrici (8) who produced anaphylactic reactions in rabbits given a second injection of an extract of *Rhizopus nigricans*.

The only organism examined in this study that failed to produce fever was *Cryptococcus neoformans*. This observation is of interest because, in contrast to other disseminated fungus infections, there is but little fever in disseminated cryptococcosis (9). The cryptococcus also differs from the other fungi in this study in two other respects: *1*) its failure to form mycelium, and *2*) its production of an enormous capsular polysaccharide. There is a possibility that the capsule may interfere with the pyrogenicity of the yeast cell by enclosing the pyrogenically active material. This idea is supported by reports that another encapsulated gram-positive organism, the pneumococcus, is also not pyrogenic upon intravenous injection of animals (10); and by our observation that a pyrogen appeared in supernates of cryptococcal suspensions after the cryptococcal cells were disrupted.

In addition to the classical pyrogenic response beginning after a short afebrile period, living fungi also produced a delayed response that sometimes appeared after a 24 hour incubation period. Such delayed fever was observed mainly with *Candida albicans* and persisted until the animal recovered or died. The kidneys and heart of these animals were studded with mycotic abscesses described in fatal human cases (6). This progressive fever following a protracted lag (or incubation) period appears to be unique to infection since it was never observed with dead fungi. The relationship between the progressive fever associated with fungus infection and the fever following injection of dead fungi remains to be determined. Because the protracted fever of candida infection (Figure 1) was accompanied by sterile blood cultures, and because a transferable pyrogen was present in sera from febrile animals, it is possible that either the fungus pyrogen or an endogenous pyrogen (11, 12) is released from the heavily infected abscesses of the kidneys and other organs.

SUMMARY

The mechanism of fever in fungus infections was studied by systematically examining pathogenic fungi for their capacity to induce fever in rabbits. Intravenous injections of viable *Candida albicans, Blastomyces dermatitidis, Histoplasma capsulatum,* and *Sporotrichum schenckii* produced fever, but cultures of *Cryptococcus neoformans* did not. The fever from these live cultures took two forms, depending upon the dose and the species of fungus: *1*) An immediate fever resembling that produced by gram-negative bacterial lipopolysaccharides. This fever began 1 to 3 hours after injection, lasted 7 to 8 hours, and sometimes produced *biphasic* curves with fever peaks of 1.5 to 4° F at 1 and 3 hours. *2*) A delayed fever, beginning as late as 24 hours after inoculation and persisting until death or recovery from infection.

The first type of fever curve was attributed to a heat-stable filterable fungus pyrogen, since the fever curve could be reproduced with autoclaved fungus cells or Seitz filtrates of fungus cultures. Unlike gram-negative bacterial pyrogens the fungus pyrogens did not cause tolerance to fever upon repeated daily injections and single massive doses did not produce a shock-like state. Instead,

one or more injections of the fungus pyrogen led to increased susceptibility to its toxic effects that produced death from hemorrhagic pneumonia. Aside from pyrogenicity, the main similarity between fungus pyrogens and the pyrogens of gram-negatives, was the marked instantaneous neutropenia and subsequent neutrophilia that followed intravenous injection.

The second type of fever curve (i.e., the delayed persistent type) was believed to be a consequence of infection of the tissues. It was observed with experimental candidiasis and sporotrichosis and was not accompanied by positive blood cultures of the infecting fungus. For this reason, and because a circulating pyrogen was found on the second day of infection, it is believed that either the fungus pyrogen or an endogenous tissue pyrogen is released from the infected tissues.

The absence of fever after injection of cryptococci coincides with the clinical observation that this fungus disease alone is often characterized by little or no fever in the course of severe systemic human infections.

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MECHANISM OF THE ALTERED BLOOD PRESSURE RESPONSIVENESS PRODUCED BY CHLOROTHIAZIDE *

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Hypertensive patients respond to chlorothiazide with a reduction of basal blood pressure while normotensive subjects do not (1, 2). This is in contrast to other known antihypertensive agents which lower blood pressure whether it is elevated or normal. The specificity of chlorothiazide raised the possibility that it might be a metabolic antagonist of the unknown pressor mechanisms operative in hypertension. Such a position has been taken by Wilkins, Hollander and Chobanian (2, 3). Evidence from this and other laboratories, however, indicates that the initial antihypertensive effect of chlorothiazide is a consequence of plasma and total extracellular fluid contraction resulting from the saluretic action of the drug (1, 4, 5). The evidence supporting this view follows. *1)* The period of falling blood pressure parallels the salt loss and plasma volume reduction and does not precede or follow it (5, 6). *2)* Massive administration of salt (average 25 g per day), sufficient partially to overcome the saluretic effect of the drug, tends to reverse the antihypertensive effect (6). *3)* Restoration of plasma volume by intravenous dextran infusions, either salt-containing or salt-free, results in partial reversal of the antihypertensive action (5, 7, 8). The failure of the normotensive subject's blood pressure to fall after chlorothiazide could be due to a lesser saluresis and consequent reduction in plasma volume or to some other mechanism. The present report is concerned with the further elucidation of this problem.

METHODS AND MATERIALS

The subjects were normotensive patients in the Mt. Alto Veterans Administration Hospital, Washington, D. C., who were convalescing from a variety of illnesses not involving the circulatory system (Table I). All patients were ambulatory and asymptomatic at the time the studies were carried out.

Twenty-four-hour urine samples were collected and analyzed for sodium and potassium in 6 subjects as described in a previous communication (8). Body weight was recorded daily for 3 days before and 3 days after chlorothiazide administration. The dosage of the drug was 0.5 g orally 3 times daily in all instances. All subjects were placed on a diet containing 500 mg salt per day and in addition were given 3.5 g of salt in tablet form in order to assure a constant salt intake during the study period.

Reactivity to levarterenol (Levophed) was tested in 14 additional subjects who were given the same diet, salt intake and chlorothiazide dosage as indicated above. In 10 of these 14 subjects the response to trimethaphan (Arfonad) also was estimated. Reactivity to levarterenol and trimethaphan was determined as follows. Both agents were diluted in 5 per cent dextrose in water to make up final concentrations of 4 mg levarterenol in 1,000 ml and 500 mg trimethaphan in 500 ml. These were given intravenously by the method previously described (9). Control titrations were carried out on the day prior to chlorothiazide administration and the experimental infusions on the third day following chlorothiazide. Immediately thereafter 500 ml of 6 per cent dextran in 5 per cent glucose in water was administered intravenously over a period of 15 to 20 minutes. The infu-

TABLE I

Cumulative negative balances (urinary losses only) of sodium and potassium and decrease in body weight in normotensive subjects given 1.5 g chlorothiazide daily

		After 3 days' chlorothiazide		
		Cumulative neg. balance		
Patient	Age	Na	K	Decr. in body wt.
	yrs	*mEq*		*kg*
C.T.	56	208	100	1.6
R.R.	28	258	52	1.8
W.S.	36	292	70	2.3
H.B.	58	185	130	0.4
C.M.	28	474	153	1.3
P.K.	35	308	144	4.8
Mean		287	108	2.0
SD		±103	±41	±0.7

* Supported in part by Grant H-720 from the National Heart Institute, Bethesda, Md. and by a grant from Merck, Sharp and Dohme, West Point, Pa.

TABLE II

Mean arterial pressure, heart rate and hematocrit before and after chlorothiazide in 14 normotensive subjects*

Subject	Mean* arterial pressure		Heart rate per min		Hematocrit	
	Control	After drug	Control	After drug	Control	After drug
	mm Hg				%	
E.F.	93	88	65	72	52.0	56.0
G.T.	84	91	69	73	40.0	42.0
J.D.	100	100	69	68	45.8	50.5
R.M.	89	103	68	66	38.5	45.0
J.B.	91	91	78	82	41.5	47.0
R.W.	93	93	80	86	45.2	49.8
F.C.	109	105	69	83	48.0	54.0
E.C.	83	82	75	90	37.0	41.5
D.P.	91	91	72	83	45.8	46.8
L.L.	97	98	78	86	48.5	50.8
D.I.	98	90	65	68	47.2	51.8
G.B.	100	110	72	84	42.5	48.5
B.A.	102	99	84	95	46.0	52.0
S.D.	95	95	64	76	43.8	38.2
Average	94.7	95.5	71.8	79.5	44.4	48.0

* "Mean" arterial pressure: $\frac{\text{systolic} + \text{diastolic}}{2}$.

sions of levarterenol and trimethaphan were again repeated as soon as the administration of dextran was completed. Thus, levarterenol and trimethaphan were infused at the same rates on three separate occasions: before chlorothiazide, following chlorothiazide, and after chlorothiazide plus dextran. Following each levarterenol infusion the blood pressure was allowed to return to the basal level and remain there for at least 10 minutes before the trimethaphan infusion was begun.

RESULTS

Sodium and potassium losses; body weight changes. Chlorothiazide produced essentially the same degree of sodium loss in the six nonedematous normotensive subjects studied as in nonedematous hypertensive patients (1, 8). The "cumulative negative balances" (8) of sodium following three days of continuous treatment with 1,500 mg of chlorothiazide daily averaged 287 (SD 103) mEq (Table I). The average loss of potassium was 108 (SD 41) mEq. The mean decrease in body weight was 2.0 (SD 0.7) kg.

Basal blood pressure and heart rate. The basal blood pressure in the 14 normotensive subjects was essentially unchanged following chlorothiazide. The "mean" (systolic + diastolic/2) blood pressure averaged 94.7 before and 95.5 mm Hg after the drug (Table II). This is in contrast to the reductions of basal blood pressure previously observed in hypertensive patients treated with chlorothiazide (1, 2). The average systolic pressure in the present group of normotensive subjects decreased slightly from 118 to 114 mm Hg and the diastolic increased from 71 to 77 mm Hg possibly reflecting the decrease in plasma volume and compensatory vasoconstriction. The average heart rate increased slightly from 72 to 79.5 beats per minute (Table II).

Effects of chlorothiazide on pressor response to levarterenol infusion before and after dextran. The same rate of infusion of levarterenol was carried out on three occasions: *1*) during the control period, *2*) after chlorothiazide, and *3*) after restoration of plasma volume depletion with 6 per cent dextran in 5 per cent glucose and water. Effective infusion rates of levarterenol varied between 0.14 and 0.45 μg per kg per minute in different subjects. In eight patients, the responses to several levels of levarterenol infusion were obtained. However, only the responses to the highest infusion rate employed in each of these cases were used for statistical analysis (Table III).

During the control or prechlorothiazide period the mean elevation of systolic blood pressure following the infusion of levarterenol was 26.6 per cent (Table III). After chlorothiazide the response of the systolic pressure to levarterenol was significantly less than in the control period (mean rise, 17.9 per cent; $p < 0.02$). Following dextran the responsiveness to levarterenol again increased, the elevation of systolic blood pressure averaging 28.4 per cent. Thus, dextran administration was followed by a significant change in the response of the systolic pressure to levarterenol in the postchlorothiazide period ($p < 0.02$).

The elevation of diastolic pressure following levarterenol averaged 34.4 per cent in the control period (Table III). After chlorothiazide the mean rise in diastolic blood pressure during the same rates of levarterenol infusion was reduced significantly (mean rise, 18.9 per cent; $p < 0.01$). The administration of dextran partially restored diastolic responsiveness, the mean elevation of diastolic pressure being 28.3 per cent. As compared with the levarterenol response immediately preceding dextran, the change was significant at the 0.05 level.

Effects of chlorothiazide before and after dextran on depressor responses to trimethaphan. Ef-

TABLE III

*Per cent changes in systolic and diastolic blood pressure to infusions of levarterenol and trimethaphan before and after chlorothiazide and after chlorothiazide plus dextran**

	(A) Before chlorothiazide	(B) After chlorothiazide	p value,† (B) compared to (A)	(C) After chlorothiazide plus dextran	p value, (C) compared to (B)
Response to levarterenol					
No. of subjects	14	14		14	
Systolic blood pressure					
Mean change (%)	+26.6	+17.9		+28.4	
SD	8.8	8.3	<0.02	11.6	<0.02
Diastolic blood pressure					
Mean change (%)	+34.4	+18.9		+28.3	
SD	13.4	12.0	<0.01	9.1	<0.05
Response to trimethaphan					
No. of subjects	10	10		10	
Systolic blood pressure					
Mean change (%)	−8.5	−14.9		−10.9	
SD	4.8	11.2	>0.05	7.6	>0.05
Diastolic blood pressure					
Mean change (%)	−7.0	−11.1		−7.6	
SD	7.7	14.9	>0.05	6.4	>0.05

* Tests were paired in that infusion rate of levarterenol or trimethaphan utilized in any one test in A was duplicated in B and C.

† Value calculated as follows:

$$\text{SE (A–B)} = \sqrt{\frac{\text{SD (A)}^2}{\text{n(A)}} + \frac{\text{SD (B)}^2}{\text{n(B)}}} = \text{standard error of the difference}$$

$$t = \frac{\text{mean (A)} - \text{mean (B)}}{\text{SE (A–B)}}.$$

fective rates of infusion of trimethaphan varied between 0.03 and 0.11 mg per kg per minute. The modifying effects of chlorothiazide and chlorothiazide plus salt-free dextran on the blood pressure responsiveness to trimethaphan were studied in 10 subjects (Table III). The mean reduction of systolic blood pressure was 8.5 per cent in the control period and 14.9 per cent after chlorothiazide to the same levels of trimethaphan infusion. Following dextran the fall in systolic pressure during trimethaphan averaged 10.9 per cent. The mean decrease in diastolic pressure during trimethaphan infusion was 7.0 per cent in the control period, 11.1 per cent after chlorothiazide and 7.6 per cent following dextran. Although these alterations suggested that chlorothiazide enhanced the diastolic pressure fall produced by trimethaphan, and that dextran partially reversed both the systolic and diastolic responses, the changes were not statistically significant.

Twelve of the 14 subjects exhibited a decrease in body weight greater than 0.5 kg following chlorothiazide. The mean decrease in weight for the entire group was − 1.4 kg. The hematocrits of the 14 subjects averaged 44.4 per cent before chlorothiazide and 48.0 per cent following the drug (Table II). No cause was found for the decrease, rather than the expected increase, in hematocrit of Subject S.D.

DISCUSSION

In nonedematous hypertensive patients the negative balance of sodium as estimated by urinary excretion during the first four days of treatment with chlorothiazide averaged 250 mEq (1, 8), which is similar to the loss of sodium observed in the present study in nonedematous normotensive subjects. The loss of body weight also was similar since the average weight loss was 2.0 kg in the hypertensive patients (8). Although plasma volume was not estimated directly in the present investigation in normotensive subjects, the significant elevations of hematocrit indicated the presence of hemoconcentration due to plasma volume reduction. It is probable, therefore, that the body fluid

changes were similar in both hypertensive and normotensive, nonedematous subjects.

Despite the fact that the body fluid changes were similar in the previously reported hypertensive patients and in the present series of normotensive subjects, the normotensive group failed to exhibit a reduction of basal blood pressure after chlorothiazide. The hypertensive patient under similar conditions (hospitalization with 4.25 g salt intake daily) displayed a reduction of "mean" (systolic + diastolic/2) blood pressure averaging 15 per cent less than the pretreatment level (1). It is of interest that this value is quantitatively similar to the 13 per cent reduction in the levarterenol pressor response of normotensive subjects following chlorothiazide.

The reduction of basal blood pressure in the hypertensive patient could be due to a specific antagonistic effect of chlorothiazide on the unknown pressor factors which maintain essential hypertension. But it could also be due to a nonspecific decrease in "reactivity" to any or all types of pressor influences. Evidence for the latter is provided in these studies. Reactivity to a controllable pressor stimulus, namely, levarterenol infusion, was reduced after chlorothiazide. Similar observations have been made by Merrill, Guinand-Baldo and Giordano, in hypertensive patients (10). Beavers and Blackmore found in normal dogs that chlorothiazide reduced reactivity to a variety of pressor agents (11).

Previous reports have shown that repletion of plasma volume with infusions of dextran, either salt-containing or salt-free, combats the depressor effect of chlorothiazide in hypertensive patients (5, 7, 8). The present studies indicate that restoration of plasma volume depletion with salt-free dextran combats the altered blood pressure responsiveness produced by chlorothiazide in normotensive subjects. It seems possible, therefore, that both the reduction of basal blood pressure in hypertensive patients and the altered reactivity seen in either hypertensive or normotensive subjects is due at least in part to the reduction in plasma volume.

An objection has been raised that the above concept does not explain the long-term antihypertensive effects of chlorothiazide (3, 12). After several months of continuous treatment the plasma volume often is retored despite continued reduction of blood pressure (8). However, the mechanism of action of antihypertensive agents is difficult to interpret after long continued treatment because the severity of the hypertension may abate under the effects of prolonged blood pressure control (13), and drug tolerance may enter to further confuse the picture. It seems unlikely that chlorothiazide reduces blood pressure by one mechanism initially and by an entirely different mechanism at a later time.

The failure of chlorothiazide to influence significantly the depressor response to trimethaphan was surprising in view of the clinical observations that chlorothiazide enhances the effectiveness of ganglion-blocking drugs in hypertensive patients (1, 2, 4). However, it is evident from the results in Table III that the blood pressure of the supine, normotensive subject is not very responsive to ganglion blockade. This provided a relatively insensitive test subject for the study. It is possible that significant changes would have been found if the more responsive hypertensive patients had been used.

SUMMARY

Chlorothiazide lowers basal blood pressure only in hypertensive and not in normotensive individuals. However, after chlorothiazide in normotensive subjects, reactivity is reduced to levarterenol. The saluretic response, weight loss and decrease in plasma volume following chlorothiazide are similar in hypertensive and normotensive subjects. Plasma volume replacement with salt-free dextran which tends to restore pretreatment basal blood pressure in hypertensive patients also tends to restore pretreatment responsiveness to levarterenol in normotensive subjects.

These observations suggest that basal blood pressure is lowered by chlorothiazide specifically in the hypertensive patient because the reduction in plasma volume produced by the drug diminishes blood pressure responsiveness to the unknown pressor mechanisms operative in that disorder. This concept does not rule out the possibility that additional factors may contribute to the antihypertensive effects of chlorothiazide and other salt-depleting procedures.

ACKNOWLEDGMENTS

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THE EFFECT OF FASTING ON THE LIPOPROTEIN LIPASE ACTIVITY OF RAT HEART AND DIAPHRAGM *

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Recent studies have indicated that the activity of lipoprotein lipase in rat adipose tissue is related to the nutritional state of the animal. Fasting induces a fall in the activity released from surviving tissue by heparin and in the activity of homogenates of adipose tissue, while glucose and insulin *in vitro* increase the heparin effect on the release of lipase from surviving adipose tissue of fasted rats. Glucose and insulin also prevent the fall in activity that occurs when tissue from fed or fasted animals is pre-incubated in buffer before preparation of the homogenates (1).

Previous observations by Zemplényi and Grafnetter suggest that the lipolytic activity of rat myocardium increases on fasting (2), and since extracts of heart tissue contain lipoprotein lipase (3) it was considered of interest to study the effect of fasting on the activity and release of this enzyme from heart tissue. The possibility that similar activity might be found in another repeatedly contracting muscle, diaphragm, was also explored.

METHODS

Male albino rats (150 to 200 g) were anesthetized with sodium pentobarbital and the heart or diaphragm excised and trimmed, rinsed quickly with Krebs-Ringer phosphate buffer and then sliced or homogenized.

In the experiments in which slices of either heart or diaphragm were used the following procedure was followed. The diaphragm was cut into two approximately 100 mg pieces; each piece was then placed in a separate flask. The heart was opened with scissors and then sectioned with a razor blade into multiple small slices, each weighing 25 mg or less. Two groups of slices, each approximately 100 mg in total weight, were then placed in separate flasks. The time from excision of the tissue until the slices were placed in the incubation medium rarely exceeded 2 minutes. To one of each pair of flasks was added 125 μg of heparin per ml (Liquaemin, Organon, 10 mg per ml). The incubation medium (unless specified otherwise) consisted of 4.5 parts Krebs-Ringer phosphate buffer, pH 7.4, 4.5 parts normal human fasting serum, and 1 part 5 per cent coconut oil emulsion (Ediol, Schenlabs). In all experiments the phosphate buffer contained 5 per cent of human albumin, the albumin having been freed of fatty acids by extraction with an acidified 4:1 isopropanol:heptane mixture (4), washed with absolute alcohol, dried, and dissolved in water with adjustment of pH to 7.4 with sodium hydroxide.

Incubation was carried out in a Dubnoff shaker at 37° C under air. At the end of 1 hour the tissue was removed, weighed, and the free fatty acid content of a sample of the medium determined by the method of Dole (4). Incubation of the medium was continued for another hour when the medium was again sampled. The activity of the lipase in the medium was estimated by the change in the free fatty acid content of the medium over the second hour; this was expressed as microequivalents of fatty acid produced per gram of tissue per hour. On occasion, incubation of the medium alone was attended by a small rise in fatty acid content. When this occurred, this change was subtracted from that occurring in the vessels containing tissue.

In experiments in which homogenates were used, approximately 100 mg of heart or diaphragm was minced, weighed, and then crushed in a glass homogenizer containing 5.5 ml of the same medium used in the studies on intact tissue; 4 ml of this mixture was then incubated for 2 hours under the conditions described above. The mixture was kept frozen from the time the tissue was placed in the medium until the start of incubation, except during the brief period required for homogenization. The lipolytic activity was determined by the change in free fatty acid content of the mixture during incubation and this was expressed as microequivalents of fatty acid produced per gram of tissue per hour.

RESULTS

The release of lipolytic activity from slices of rat heart and diaphragm. The release of lipolytic activity from pieces of heart and diaphragm of fed and fasted rats and the effect of heparin on this activity are illustrated in Figure 1. In each

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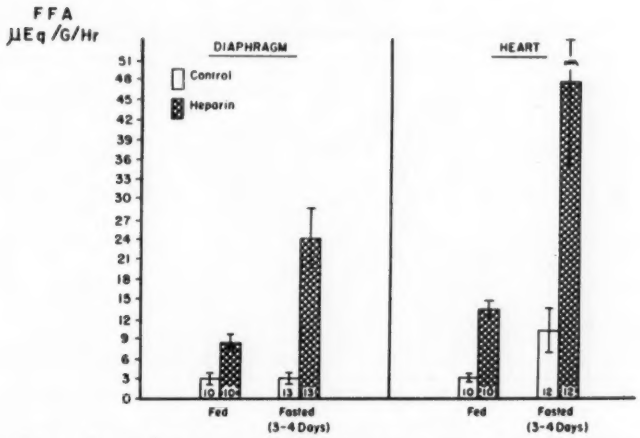

FIG. 1. THE RELEASE OF LIPOPROTEIN LIPASE FROM SLICES OF RAT HEART AND DIAPHRAGM. Statistical analysis: control vs. heparin: $p < 0.01$ in each instance. Control fed vs. control fasted heart: $0.05 < p < 0.1$. Heparin effect on fed vs. fasted diaphragm: $p < 0.01$. Heparin effect on fed vs. fasted heart: $0.01 < p < 0.05$. Brackets: SE. Number of animals indicated at foot of each column in this and succeeding figures.

instance the effect of heparin was to increase the lipolytic activity in the medium, as shown during the second hour of incubation when tissue was no longer present. When the animals were fasted for 72 to 96 hours the heparin effect with both tissues was significantly increased, while a fast of 48 hours produced a smaller change. As reported by Cherkes and Gordon (5), a fasting interval of 24 hours or less did not alter the heparin response. One M sodium chloride, a known inhibitor of lipoprotein lipase (3), substantially reduced the activity that had been released by heart and diaphragm slices from fed or fasted animals (Table I).

TABLE I

*Inhibition by 1 M NaCl of lipase released by heart and diaphragm slices**

Tissue	Per cent inhibition
Fed diaphragm	70
Fasted diaphragm (3 days)	96
Fed heart	88
Fasted heart (3 days)	94

* Two groups of slices from each tissue were incubated separately in a medium of 1.8 ml serum, 0.45 ml albumin and 125 μg/ml heparin for 1 hour. Tissue was then removed and 1.35 ml phosphate buffer added to one flask, and 1.35 ml of the buffer containing 0.156 g/ml NaCl to the other. After 5 minutes of incubation at room temperature, 0.4 ml of 5% coconut oil was added to all flasks and the free fatty acid change measured over an ensuing hour of incubation.

These results indicate that a lipase similar to lipoprotein lipase was released from slices of rat heart and diaphragm. It has been demonstrated that heparin added *in vitro* causes a release of lipoprotein lipase from adipose tissue (1, 5), and it is likely that a similar process occurred in the present experiments. It is difficult, however, to attribute the effect of heparin solely to an effect on enzyme release; in experiments using heart tissue from fasted rats, heparin produced a two- to threefold increase in the lipolytic activity of the medium when it was added only after the tissue had been removed. This is analagous to the effect of heparin on partially purified enzyme preparations (3). It is therefore probable that both processes, increased release of enzyme and activation of enzyme in tissue and medium, contributed to the total response to heparin noted.

Lipolytic activity of homogenates of rat heart and diaphrgam. The effect of fasting on the lipolytic activity of homogenates of rat heart and diaphragm is illustrated in Figure 2. The activity of heart homogenates was consistently greater than that of diaphragm while the activity of both tissues increased progressively as fasting was

TABLE II

*The effect of pre-incubation on the lipolytic activity of homogenates of heart and diaphragm from four-day fasted rats**

Diaphragm				Heart			
Initial†		3-Hour‡		Initial		3-Hour	
Serum§	No serum‖	Serum	No serum	Serum	No serum	Serum	No serum
144	93	62	50	273	130	108	65
53	28	26	32	366	139	173	122
76	22	32	27				
88	23	23	18				
Mean 90	42	36	32	319	134	140	93

* Lipolysis expressed as microequivalents per gram per hour.
† Tissue homogenized immediately on removal from animal.
‡ Tissue incubated in phosphate buffer for 3 hours and then homogenized.
§ Serum: the medium consisted of 4.5 parts serum, 4.5 parts phosphate buffer with 5% albumin and 1 part 5% coconut oil.
‖ No serum: the medium consisted of 9 parts phosphate buffer with 5% albumin and 1 part 5% coconut oil.

prolonged. The greater effect of heparin when tissue slices from fasted animals were used was thus probably a reflection of the increased activity of lipoprotein lipase in the tissue. These findings are in contrast to those with adipose tissue in which fasting induced a fall in the lipoprotein lipase activity of the tissue homogenates (1).

Since the optimal substrate of lipoprotein lipase is a lipoprotein-triglyceride mixture (6), an attempt was made to characterize the activities of heart and diaphragm homogenates by noting the effect on these activities of omitting serum, and hence lipoprotein, from the medium. In these experiments one piece of tissue was homogenized in the medium described above, while a second piece was ground in a medium of similar composition except for the absence of serum; the total volume of the medium and the concentration of coconut oil was the same in both instances. The results are shown in Figure 3. Addition of serum to the coconut oil substrate increased the activity of heart homogenates from fed and fasted animals

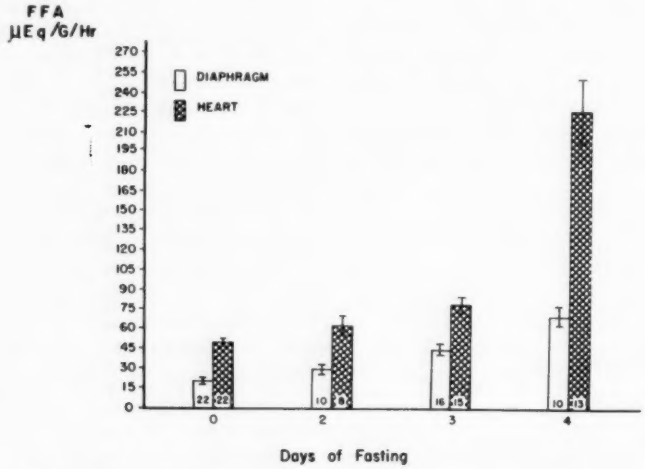

FIG. 2. LIPOLYTIC ACTIVITY OF HOMOGENATES OF HEART AND DIAPHRAGM. Statistical analysis: (Diaphragm) fed vs. 2-day fast: $0.01 < p < 0.05$; fed vs. 3- or 4-day fast: $p < 0.01$. (Heart) fed vs. 2-day fast: $p > 0.1$; fed vs. 3- or 4-day fast: $p < 0.01$.

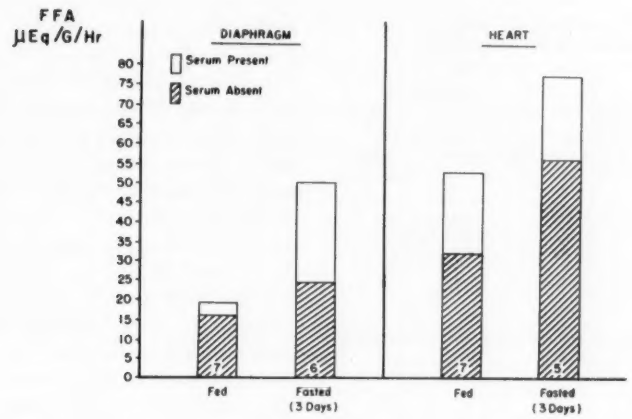

FIG. 3. LIPOLYTIC ACTIVITY OF HOMOGENATES OF HEART AND DIAPHRAGM WITH AND WITHOUT SERUM IN THE MEDIUM.

and of diaphragm homogenates from fasted rats. It had little effect when diaphragm tissue from fed rats was used. As shown in Table II, the activity of both tissues from 4-day fasted animals fell when the intact tissues were incubated in buffer for 3 hours before homogenizing. At the end of this incubation, heart tissue was still considerably more active with the serum-coconut oil substrate but diaphragm only slightly so.

Inhibition by protamine was studied by adding 900 μg per ml of protamine to a homogenate of tissue in phosphate buffer and albumin. This mixture was shaken at room temperature for 5 minutes and then an aliquot of the mixture was added to serum and coconut oil, the ingredients being present in the ratios previously described.

TABLE III

*The effect of protamine on the lipolytic activity of rat heart and diaphragm**

Tissue homogenized in	Diaphragm		Heart	
	Fed	Fasted†	Fed	Fasted
Complete medium‡		98		371
Buffer-albumin§	11	29	34	265
Buffer-albumin§ + protamine	12	29	11	101

* Lipolysis expressed as microequivalents per gram per hour.
† Four days.
‡ Incubated immediately at 37° C.
§ Incubated at room temperature for 5 minutes, remaining medium then added and incubation at 37° C commenced.

A control specimen was treated in the same way without protamine. In some instances a third piece of the same tissue was homogenized in the entire medium and incubated immediately. Sampling and incubation were carried out as described for the other homogenate experiments. As demonstrated in Table III, protamine consistently inhibited the lipolytic activity of homogenates of heart tissue prepared from either fasted or fed animals, but its effect on diaphragm was negligible. As is also shown in this table, homogenates of heart or diaphragm from fasted animals rapidly lost activity when allowed to stand for short periods before introduction of substrate.

To determine whether fasted tissue contained factors that might increase the activity of fed tissue, or conversely, whether homogenates of fed tissue would inhibit the activity of homogenates from fasted animals, the activity of mixtures of tissues from fed and 4-day fasted animals was compared with activity predicted on the basis of the behavior of each tissue alone. The results summarized in Table IV indicate that activation of fed tissue by fasted tissue or inhibition of fasted tissue by fed tissue could not be demonstrated by this means.

DISCUSSION

The characteristics of the lipolytic activity of homogenates of heart tissue from fed and fasted animals are similar to those noted by Korn in

TABLE IV

Predicted and observed lipolytic activity of fed and fasted tissues homogenized together

Tissue	No. of expts.	Lipolysis*	
		Predicted	Observed
Diaphragm	4	4.8±0.7†	4.2±0.5
Heart	3	12.2±1.5	11.3±0.3

* Lipolysis expressed as microequivalents of free fatty acid produced. The medium consisted of 4.5 parts serum, 4.5 parts phosphate buffer with 5% albumin and 1 part 5% coconut oil.
† Standard error.

extracts of rat heart tissue (3), suggesting that lipoprotein lipase provides the major portion of the lipolytic activity of heart tissue. The greater effect of heparin when heart tissue from fasted animals was used suggests that the increase in lipolytic activity of heart homogenates with fasting is due mainly to an increase in lipoprotein lipase activity.

The nature of the activity of diaphragm, however, is not so clear. There is undoubtedly some lipoprotein lipase activity present in diaphragm which rises with fasting, as is evidenced by the heparin effect on tissue from fed animals and the increase in this effect with fasting. The failure of the serum-triglyceride mixture to serve as a better substrate than the triglyceride alone when homogenates of diaphragm from fed animals or from fasted animals that had been pre-incubated were used, suggests that in these instances the major activity may be of a type other than lipoprotein lipase. Moreover, no evidence of inhibition by protamine was observed in homogenates of diaphragm as there was just as great a reduction in activity without protamine as with it during the pre-incubation interval. This might be explained by a greater lability of lipoprotein lipase than of the residual activity and by the finding that the latter is not susceptible to inhibition by protamine.

The factors that induce the changes in lipoprotein lipase activity on fasting in heart and diaphragm remain to be elucidated. Whatever these factors, the changes produced are sufficiently striking to suggest that they may play an important role in the adaptation of the organism to fasting.

The process by which chylomicrons are taken up by various tissues has not as yet been clearly defined. It is possible that, at least in some tissues, hydrolysis of the triglyceride is a necessary accompaniment of tissue uptake. Since the optimal substrate of lipoprotein lipase is a lipoprotein-triglyceride complex such as chylomicrons (6), changes in the activity of this enzyme in various areas may well affect the tissue distribution of circulating triglyceride. Recent studies by Bragdon and Gordon (7) have shown that fasting induces in the rat profound alterations in the tissue distribution of labeled chylomicrons. When the chylomicrons were administered following a 20-hour fast, less of the label was found in adipose tissue and more in heart tissue than when fed animals were used. These changes parallel in direction the changes in lipoprotein lipase activity induced by fasting in these two tissues, although they precede the demonstrable change in heart activity by 24 to 48 hours. If lipoprotein lipase aids in the tissue uptake of chylomicrons, the fall in activity of this enzyme in adipose tissue with fasting would permit the fasted organism to divert more of the circulating triglyceride to tissues such as heart where this nutrient could be readily taken up. As prolongation of fasting is associated with a progressive rise in lipoprotein lipase activity in diaphragm and heart, a more extensive removal of chylomicrons from the circulation by these tissues may occur as fasting progresses.

SUMMARY

Slices of rat heart and diaphragm were incubated for one hour in a triglyceride medium with and without heparin; the slices were then removed and the lipolytic activity of the medium measured over the ensuing hour. Heparin significantly increased the activity in the medium both with tissues from fed or fasted rats, the effect of heparin being greatest when animals fasted for three to four days were used.

Homogenates of heart tissue had greater lipolytic activity than did those of diaphragm, the activities of both tissues increasing progressively with fasting. The characteristics of the activity of heart homogenates were similar to those of lipoprotein lipase while the characteristics of diaphragm differed in certain respects.

It is suggested that the activity of lipoprotein lipase in various tissues may, in part, determine the tissue uptake of circulating triglyceride. The similarity in the direction of change produced by fasting in the distribution of isotopic label from chylomicrons and in the lipoprotein lipase activity in rat heart and adipose tissues supports this view.

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DIRECT MEASUREMENT OF CHOLESTEROL ABSORPTION VIA THE THORACIC DUCT IN MAN *

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The route of absorption of steroids from the gastrointestinal tract seems to be largely determined by the chemical nature of the compounds. Methyl testosterone (1), 17α-methyl estradiol (2), and cortisone acetate (3) are absorbed in the rat by way of the portal circulation. Studies from this laboratory in human subjects showed that testosterone, cortisol, cortisone acetate, and corticosterone are also absorbed in this manner and are virtually absent from the lymph (4). In contrast, absorption of cholesterol into lymph of the rat (5, 6) and dog (7) accounts for essentially all of the sterol that enters these species from the diet. Although it might be anticipated from these animal studies and from the nonpolar nature of the molecule that cholesterol would be absorbed via the lymph in human subjects, it was of interest to test this directly, since some aspects of steroid metabolism in the laboratory animal are different from those observed in man (8–10). By means of cannulation of the thoracic duct and the administration of labeled cholesterol, it has been possible for the first time to study directly the absorption and esterification of cholesterol in man. These studies have demonstrated that dietary cholesterol is largely absorbed through the lymphatics and that the major portion is esterified during the absorptive process.

MATERIAL AND METHODS

The clinical data concerning the subjects are given in Table I. A polyethylene catheter was inserted into the left thoracic duct at operation as described previously (4). The lymph was continuously collected from the thoracic duct cannula at predetermined time intervals and blood samples were withdrawn periodically. The patients were maintained in fluid and protein balance by the infusion of appropriate quantities of electrolytes and salt-free albumin during the period that large volomes of lymph were being removed.

Cholesterol-14, 15-H^3 and sodium acetate-2-C^{14} were given to 1 subject while cholesterol-4-C^{14} was administered to 3 other patients. The radioactive compounds were given orally and the amounts are listed in Table I. Radiochemically pure free and ester cholesterol were isolated from plasma and red cells by methods that have been described (11). Free and ester cholesterol were also obtained from lymph by the same procedures, but in a few instances lymph was deproteinized with acetone-alcohol, the solution was concentrated, and the portion of the residue that was soluble in petroleum ether was chromatographed on alumina to isolate free cholesterol and cholesterol esters.

C^{14} radioactivity was determined by counting cholesterol digitonide mounted on stainless steel planchets in a windowless gas-flow counter (12). C^{14} activity was also measured by combustion of cholesterol digitonide and counting the carbon dioxide (13); tritium was determined in the same sample by reducing the water produced by the combustion to hydrogen and counting in the proportional region (14).

RESULTS

Figure 1 shows the specific activity of lymph and plasma cholesterol-C^{14} observed over 75 hours following the oral administration of cholesterol-4-C^{14} to Subject W.I. The lymph free and ester cholesterol attained a peak specific activity at 8 hours. It should be noted that ester cholesterol appeared promptly in the lymph although free cholesterol was administered. The specific activity of the lymph ester cholesterol is equal to or greater than that of the free cholesterol, especially during the first 30 hours of the experiment. Cholesterol analyses of this lymph at various intervals showed that an average of 65 per cent of the sterol was esterified (Table I). Since there was a greater total amount of ester cholesterol of high specific activity in the lymph of Subject W.I., esterification of cholesterol must have been achieved in the intestinal mucosa. The spe-

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TABLE I

Clinical data and administration of radioactive compounds

Subject, age*	Diagnosis, cancer	Lymph cholesterol (avg.) Total	Lymph cholesterol (avg.) Ester	Lymph flow	Compound administered	Wt. of comp.	Radioactivity
		mg %	*%*	*ml/hr*		*mg*	*dpm† $\times 10^{-7}$*
W.I., 55	Larynx (laryngectomy)	80	68	48	Cholesterol-4-C^{14}	13.6	14.3
M.O., 70	Tonsil	60	55	59	Cholesterol-4-C^{14}	41.0	4.4
P.U., 70	Tonsil			58	Cholesterol-4-C^{14}	11.2	11.8
W.E., 55	Lip	62	52	70	Cholesterol-14, 15-H^3	7.5	21.8
					Sodium acetate-2-C^{14}	16.9	44.0

* All were men.
† Disintegrations per min.

cific activity relationships of free and ester lymph cholesterol can perhaps be explained by the experiments of Swell and colleagues (15) who fed cholesterol-4-C^{14} to rats and found that the radioactive cholesterol present in the intestinal mucosa was largely in the free form. This would suggest that esterification occurs in conjunction with the transport of the newly absorbed cholesterol from the cells of the mucosa into the lymph. Therefore, the relative quantity of the mucosal cholesterol which is esterified would largely determine the proportion of lymph free and ester cholesterol as well as their specific activities.

During the period of lymph collection, over 27 per cent of the radioactive cholesterol was absorbed by this route (Table II). The lower set of curves in Figure 1 shows the specific activity of plasma cholesterol observed simultaneously with that in lymph. During the period of active absorption into the lymph, the plasma cholesterol attained a specific activity of approximately 1.5

TABLE II

Distribution of radioactivity from labeled cholesterol

Subject, age	Max. incorp. radioactivity into plasma	Cumulative radioactivity in lymph	Cumulative radioactivity in feces
	%*	%	%
W.I., 55	1.0[72]†	27.4[72]	
M.O., 70	0.3[7]‡	4.5[44]	
P.U., 70	2.0[24]	27.1[84]	42[144]
W.E., 55	1–2[10]‡	7–14[10]	

* Percentage of administered radioactive cholesterol.
† Elapsed time in hours, [ ].
‡ Calculated from specific activity of plasma cholesterol and size of plasma and liver cholesterol pool (16). All other values by direct measurement of total radioactivity.

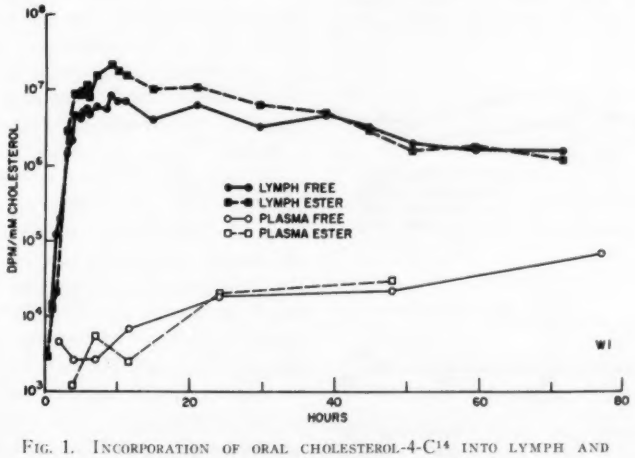

FIG. 1. INCORPORATION OF ORAL CHOLESTEROL-4-C^{14} INTO LYMPH AND PLASMA CHOLESTEROL; SUBJECT W.I.

per cent of that in the lymph. The low value for the specific activity of plasma cholesterol is due to the fact that the principal lymphatic outflow from the intestine was diverted from the body through the cannula and only small quantities of lymph could reach the circulation through collateral channels. The labeled cholesterol in this small quantity of lymph was then diluted with the pre-existing unlabeled circulating sterol pool to yield plasma cholesterol of low specific activity. The total quantity absorbed through these collateral channels approximated 1 per cent of the administered radioactivity. These values are given in Table II and can be calculated from the specific activity of plasma cholesterol and the size of the plasma and liver cholesterol pool which are known to be in isotopic equilibrium (16).

The rate curves of the specific activity of free and ester cholesterol in the plasma and lymph of Subject M.O. are presented in Figure 2. The specific activity of lymph ester cholesterol during the time of maximal absorption was about 1.5 times that of free sterol. It can be seen in Figure 2 that the specific activity of plasma sterol was less than 1 per cent of that of lymph cholesterol. About 0.3 per cent of the radioactivity was present in the circulation as compared with 4.5 per cent which appeared in the lymph that was drained off (Table II).

In Subject P.U., who also received cholesterol-4-C^{14}, the specific activity of lymph free cholesterol was higher than that of ester cholesterol, but both of these were over 50 times the

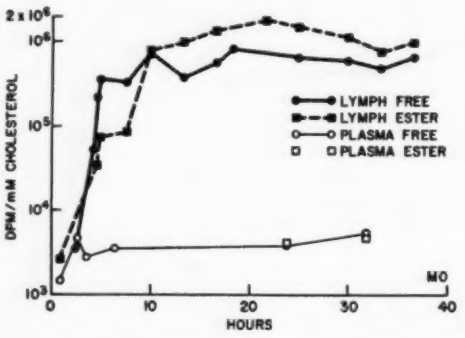

FIG. 2. INCORPORATION OF ORAL CHOLESTEROL-4-C^{14} INTO LYMPH AND PLASMA CHOLESTEROL; SUBJECT M.O.

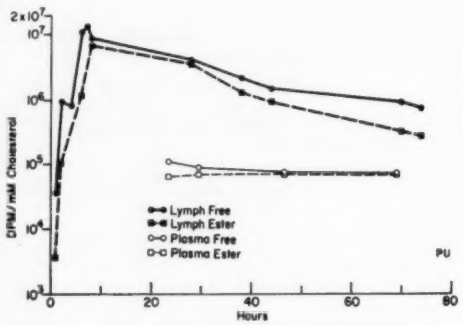

FIG. 3. INCORPORATION OF ORAL CHOLESTEROL-4-C^{14} INTO LYMPH AND PLASMA CHOLESTEROL; SUBJECT P.U.

specific activity of plasma sterol, as can be seen in Figure 3. Approximately 27 per cent of the administered dose was accounted for in the lymph, which is over 10 times that in the circulation (Table II). In this subject where measurement of fecal radioactivity was practicable, 42 per cent was eliminated by this route after six days.

Subject W.E. was given cholesterol-14, 15-H^3, and during the 10 hours in which observations were made, he absorbed approximately 10 per cent of the radioactive cholesterol in the intestinal lymph. Collection of lymph had to be discontinued at this time due to blockage in the cannula, but it can be seen in Figure 4 that the sustained peak specific activity of lymph free cholesterol indicates that cholesterol absorption continued for at least this period of time. The specific activity of cholesterol-H^3 in the plasma was approximately 0.1 per cent of that in the lymph and from this it can be calculated that 0.2 per cent of the dose appeared in the circulation (Table II).

Subject W.E. received C^{14}-acetate simultaneously with the oral dose of tritium-labeled cholesterol. This procedure permitted a study of lymphatic transport of endogenously synthesized cholesterol in order to provide comparison with the data concerning exogenous cholesterol. Figure 5 shows the specific activity of lymph and plasma cholesterol of biosynthetic origin in this subject. The free cholesterol-C^{14} of plasma has the highest specific activity through the 10-hour period of observation. The lymph free cholesterol specific activity paralleled that of the plasma but was only about one-fifth of the value. Both the

lymph and plasma ester cholesterol have similar specific activities except for the two earliest values of lymph ester cholesterol at 26 minutes and 55 minutes after administration of the precursor; these values were higher than corresponding plasma ester cholesterol specific activities. Since the only material of high specific activity available at this time is the plasma free cholesterol, it is tempting to assume that the two early samples of lymph ester cholesterol were derived by the esterification of plasma free cholesterol in the intestine. The shape and magnitude of C^{14}-labeled plasma free and ester cholesterol radioactivity curves are generally similar to those of intact subjects. This would be expected if the sodium acetate-2-C^{14} fed to the patients with cannulated thoracic ducts was absorbed into the portal circulation and transported to the site of cholesterol synthesis by a route not involving lymph. Measurement of the total radioactivity in the lymph and plasma of Subject W.E. showed that 20 minutes after ingestion of the labeled sodium acetate, the radioactivity in plasma was seven times greater than that in an equal volume of lymph; the shape of the curves shows that this difference was much larger in the initial moments of the experiment (Figure 6).

The specific activity of endogenous (C^{14}) cholesterol in the lymph was lower than the corresponding values in plasma and could be due to three factors: *1*) dilution of lymph cholesterol by unlabeled sterol from the intestinal mucosa at the start of the experiment; *2*) continuous dilution by

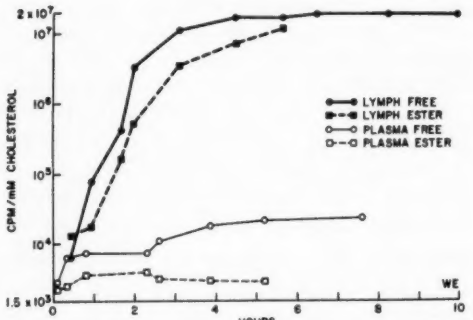

FIG. 4. INCORPORATION OF ORAL CHOLESTEROL-14, 15-H^3 INTO LYMPH AND PLASMA CHOLESTEROL; SUBJECT W.E.

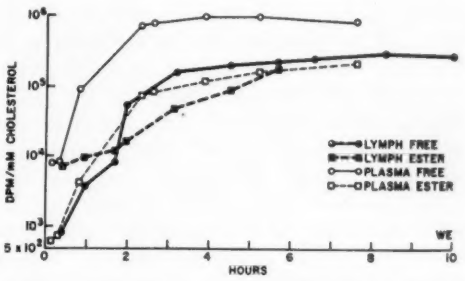

FIG. 5. INCORPORATION OF ORAL ACETATE-2-C^{14} INTO LYMPH AND PLASMA CHOLESTEROL, SUBJECT W.E.

dietary cholesterol; *3*) the possible operation of an ultrafiltration process between plasma and lymph which might remove lipoprotein containing newly labeled cholesterol.

DISCUSSION

1. Absorption of cholesterol. These studies on the direct measurement of cholesterol absorption into lymph in man are in substantial agreement with earlier results obtained in animals. The data indicate that the lymphatic system of the intestine, rather than the portal circulation, is the route through which cholesterol is absorbed. From 4 to 27 per cent of the orally administered cholesterol was absorbed in patients in this study over periods of time varying from 8 to 80 hours. In rats, from 2 to 49 per cent of the administered dose was absorbed (6, 17–19), and Blomstrand and Ahrens calculated that 20 per cent of the administered cholesterol was absorbed in a patient with chyluria (20). This relatively wide range of cholesterol absorption agrees with the range of 8 to 33 per cent previously reported from this laboratory in intact subjects (21). These variations might be anticipated because of the many factors involved in cholesterol absorption, including the amount of cholesterol (22, 23), fat and fatty acids (24, 25), presence of bile salts (17, 19, 26, 27), pancreatic secretion (17, 25, 28), and other sterols (23).

The amount of labeled cholesterol which appeared in the plasma was only a small fraction of the amount in the lymph. This result is consistent with the conclusion that lymph is the principal, if not the only, route for the transport of newly absorbed cholesterol from the intestine. It is to be

anticipated that small amounts of cholesterol would enter into the circulation despite the diversion of the major portion of the intestinal lymph from the body. It is known from anatomical and radiographic studies that a right thoracic duct as well as other auxiliary lymphatic channels transport a significant amount of lymph in many subjects (*29*). However, there is little question that the left thoracic duct was the main route of absorption in the four subjects of this investigation. It is of interest that substances of similar formal structure, but differing in polarity, such as cholesterol and steroid hormones (testosterone, cortisol) are absorbed through different routes (4).

2. Esterification of lymph cholesterol. Although free cholesterol was administered, most of the newly absorbed cholesterol in lymph was esterified. From 50 to 80 per cent of the radioactive cholesterol in lymph was present as ester, which is quite similar to values observed in rats (*6, 17, 22, 30*) and dogs (*7*). Animal studies indicate that esterification with fatty acid occurs before the appearance of newly absorbed cholesterol in the lymph (*5, 22–25, 27*). Cholesterol in the free form appears to be the material actually transported from the intestinal lumen into the mucosa. Esterification of the free sterol then takes place in the mucosa during transport into the lymph (*15, 27*). In contrast, Favarger and Metzger report that, in the dog and rat, free cholesterol was absorbed into the lymph and was esterified by esterases present in that fluid (*31*). in Figure 5, which illustrates the incorporation of

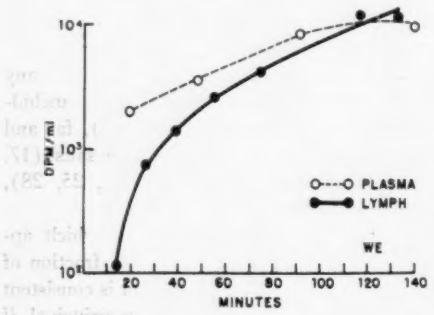

FIG. 6. TOTAL RADIOACTIVITY IN LYMPH AND PLASMA AFTER ORAL ADMINISTRATION OF ACETATE-2-C^{14}; SUBJECT W.E.

acetate into lymph and plasma cholesterol, the lymph ester cholesterol has a greater specific activity than does the corresponding lymph free cholesterol during the first two hours. Since plasma free cholesterol is initially labeled in the biosynthetic process, it seems probable that the newly synthesized radioactive cholesterol of the plasma may be esterified in the cells of the intestine and directly thereafter appears in the lymph as ester cholesterol. An alternative explanation is that some cholesterol may be biosynthesized from acetate in the intestinal wall and esterified as it appears in the lymph. In the intestinal lumen, the cholesterol is composed of a mixture of dietary sterol, cholesterol secreted from the intestinal wall, and biliary cholesterol secreted from the liver. It is realized that this is not a homogenous pool of cholesterol but one in which the relative cholesterol contribution from each source might vary; indeed, it is possible that unknown factors may be operative which permit cholesterol from each source to be absorbed or reabsorbed at different rates. Nevertheless, this study and others cited show that cholesterol in this mixture is largely esterified during absorption. Therefore, there appear to be at least two sources of ester cholesterol in the lymph: *1*) from the intestinal lumen; *2*) from the intestinal mucosa where the cholesterol may originate either by ultrafiltration from the plasma or by biosynthesis from endogenous precursors at this site. Thus cholesterol from various origins which is esterified in the intestinal mucosa may serve as a significant portion of the ester cholesterol moiety of the circulating lipoprotein. These considerations focus attention on possible alterations of the metabolic activity of intestinal mucosa in addition to the well recognized disturbances of liver function which produce changes in level of circulating cholesterol ester.

3. Clearance of lymph cholesterol. The extent of intestinal cholesterol esterification in humans may also be germane to alterations in cholesterol metabolism observed in hyperlipemic-hypercholesterolemic subjects. From our previous studies (*21*) on absorption of cholesterol in normocholesteremic subjects, it is known that the specific activity of plasma ester cholesterol was less than that of plasma free cholesterol during the first two days following the oral administration of labeled

sterol. The actual ratio of the specific activities of plasma ester to free cholesterol in normocholesterolemic subjects about 24 hours after the radioactive dose was 0.7 to 0.8. However, in hyperlipemic subjects, the same ratio was 1.6 to 2.5.[1] If it were assumed that all of the labeled ester and free cholesterol absorbed in lymph were retained in the plasma and mixed indistinguishably with plasma cholesterol, it is possible to calculate a resultant specific activity ratio. In the normocholesteremic subjects this would yield a value of ester to free of about 2.0 (assuming lymph cholesterol to be about 80 per cent ester) in contrast to the observed ratio of 0.7 to 0.8. However, the calculated ratio is close to the range of 1.6 to 2.5 observed in the plasma of hyperlipemic subjects. It should be noted that the amount of free and ester cholesterol absorbed after a tracer dose into lymph is known only for normocholesteremic subjects and might be different in the hyperlipemic. However, these relations strongly suggest that the normocholesteremic might be able to clear a portion of the newly absorbed ester cholesterol from the plasma, whereas this mechanism might not be fully operative in the hyperlipemic patient. These considerations point to the desirability of additional studies of the intestinal esterification mechanism as well as of the ester removal mechanism for the fuller understanding of pathologic hyperlipemic states.

SUMMARY

1. Cholesterol-14, 15-H^3 and sodium acetate-2-C^{14} were orally administered to one subject while cholesterol-4-C^{14} was given to three other subjects. These individuals had cannulas in the thoracic duct so that lymphatic absorption of cholesterol could be directly studied.

2. The amount of labeled cholesterol absorbed into the lymph varied from 4 to 27 per cent of the administered dose. The major portion of this absorbed cholesterol appeared in the lymph in esterified form.

3. A minor role in cholesterol absorption by the collateral lymphatics was evidenced by the low level of radioactivity in plasma cholesterol.

4. Although sodium acetate is not absorbed in the human by way of the lymph system, the amount incorporated into circulating cholesterol was approximately that found in intact subjects.

5. The absorption and esterification of cholesterol as well as the possible role of cholesterol metabolism in the intestine and lymph have been discussed with reference to pathologic hyperlipemia.

[1] Calculated from data taken from subjects in Reference 21 and other unpublished data from this laboratory.

ACKNOWLEDGMENT

The support and interest of Dr. T. F. Gallagher in this investigation are gratefully acknowledged.

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A LECITHINASE A IN DUODENAL CONTENTS OF MAN

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Lecithinase A catalyzes the removal of a fatty acid from lecithin or the cephalins to yield lysolecithin or the lysocephalins which are particularly noted for their lytic effect on red blood cells (1). Hanahan (2) studied the action of the enzyme in extracts of pancreatin on lecithin in diethyl ether. This method was used by Hanahan, Rodbell and Turner (3) and improved by Long and Penny (4) in studies of the action of the enzyme in venom on natural and synthetic phosphoglycerides. Epstein and Shapiro (5) and Rimon and Shapiro (6) measured the enzyme from rat intestinal mucosa and ox pancreas in phosphate buffer.

Borgström's (7) report of the almost complete conversion of lecithin in bile to lysolecithin in the upper small intestine of man prompted us to undertake the measurement of the enzyme involved. A simplified assay was evolved using the fasting duodenal contents of man as the source of enzyme. The release of fatty acids from lecithin was measured titrimetrically. The concurrent decrease in lecithin and equivalent increase in lysolecithin were measured by quantitative chromatography using silicic acid impregnated paper (8, 9). Some factors which increase or decrease the sensitivity of the assay were also evaluated.

EXPERIMENTAL METHODS

Duodenal contents as a source of pancreatic secretion were obtained from 5 men by aspiration. Collections varied in bile content. Lyophilized cobra venom (*Naja naja*) was obtained from the Ross Allen Reptile Institute; it was used as a source of lecithinase A only once, as specifically indicated.

Pure lecithin, lysolecithin and sphingomyelin were prepared from egg yolk by the method of Lea, Rhodes and Stoll (10, 11). Purity of the eluates used was determined by paper chromatography (8). Moles of egg lecithin were calculated as (linoleylstearoyl)-glycerylphosphorylcholine. (Dipalmitoleyl)-glycerylphosphorylcholine and (β-monostearoyl)-glycerylphosphorylcholine were provided through the kindness of Dr. D. J. Hanahan.

All evaporations were carried out with a rotary film vacuum evaporator at 40° C and approximately 18 mm Hg. Lecithin substrates were prepared by evaporation of a methanol: chloroform solution (approximately 1: 1, vol/vol) of lecithin, addition of a buffered solution of activators, and emulsification and partial solution of the mixture with a laboratory shaker.

The total release of fatty acids in the incubations was measured by the method of Dole (12). One ml aliquots were extracted immediately after addition of enzyme and at selected time intervals thereafter. A one-phase titration was obtained by preparing the indicator and alkali in absolute ethanol.

Lipid extracts for phospholipid chromatography were prepared by the addition of 1 ml of the incubation mixture to approximately 18 ml of ethanol: ether (3: 1, vol/vol), heating to boiling, and diluting with the ethanol: ether to 25 ml at room temperature. After filtering through lipid-free filter paper, a 20 ml aliquot was evaporated to dryness and the lipids dissolved in 3 ml of isoamyl alcohol: benzene (1: 1, vol/vol); 100 μl was applied to the paper for each analysis unless otherwise indicated.

The identification of phospholipids on silicic acid impregnated filter paper was made essentially using the techniques of Marinetti, Stotz, Erbland and Kochen (8, 9). The solvent system diisobutyl ketone: acetic acid: water (40: 30: 7, vol/vol) was used in this study. Quantitative estimations of the individual phospholipids were made by phosphorus analyses following their elution from the paper. Phosphorus was determined by our modification of the method of Beveridge and Johnson (13) in which 1 μg of phosphorus yields an absorbancy of 0.173 in 1 cm cells. Our recoveries were typically 85 to 95 per cent, using phospholipids applied individually or as mixtures at levels of 0.6 to 5.7 μg of lipid phosphorus. Values reported were corrected for an average 90 per cent recovery, and expressed as amount present in the total incubation mixture.

Borate buffer, 0.05 M, was prepared by adding potassium hydroxide solution to 50.0 ml of 0.2 M boric acid until the desired pH at 200 ml volume was reached. Solutions in this buffer were made prior to final pH adjustment.

Sodium deoxycholate was purified by the following procedure (14): 10 g was dissolved in 50 ml of 95 per cent ethanol, filtered, concentrated to 20 ml, and added slowly to 150 ml cold ethyl ether. The crystals were washed with ether and then dried, utilizing a vacuum evaporator as for lipid extracts, advancing the temperature to 98° C for 10 minutes.

RESULTS

Enzymatic action of duodenal contents. The similarity of action of duodenal contents and cobra venom on the lecithin and phosphatidylethanolamine of an aqueous egg yolk emulsion is shown with silicic acid chromatograms in Figure 1. This means of following the enzymatic degradation of phospholipids was then used to follow the conversion of pure lecithin to lysolecithin by duodenal contents. With egg lecithin or lecithin in blood se-

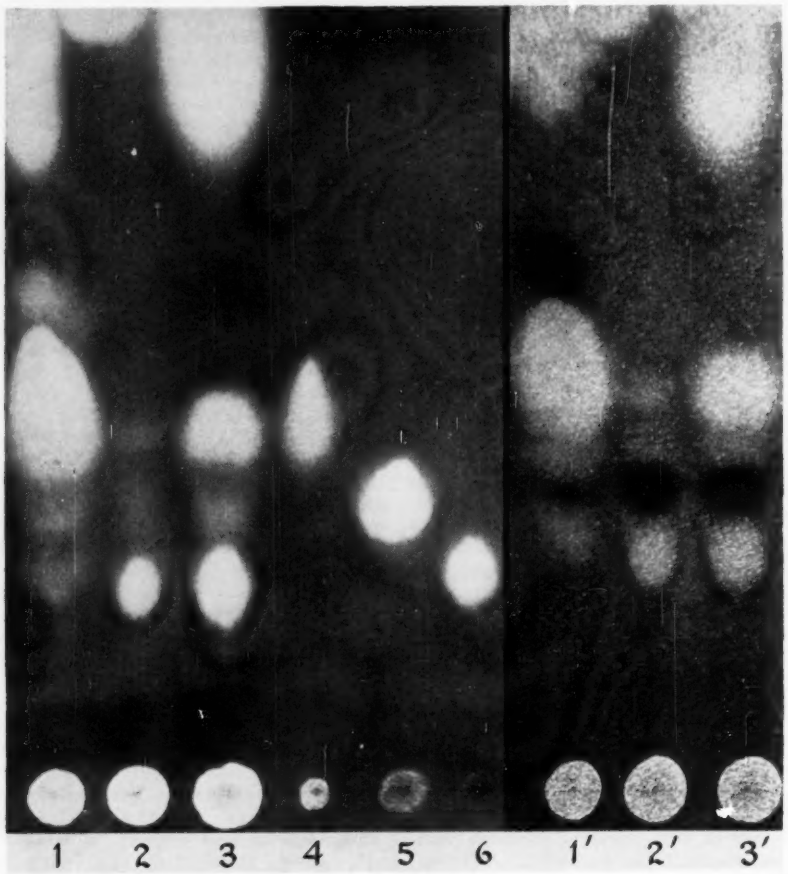

FIG. 1. THE ACTION OF COBRA VENOM AND DUODENAL CONTENTS ON EGG PHOSPHOLIPIDS. Three separate incubations for 2 hours at 38° C were made, using 5 ml of one egg yolk emulsified in 100 ml of water and adding: *a*) 1 ml of water as a control, *b*) 2 mg dried venom in 1 ml water, *c*) 1 ml of duodenal contents. After preparation of extracts, 50 μl from incubations *a* and *c* were applied at origins 1 and 3 while 25 μl from *b* was applied at 2. The amount of phospholipid contributed to 3 by duodenal drainage is negligible. Pure egg lecithin, sphingomyelin, and lysolecithin were applied at origins 4, 5, and 6, respectively. On the left the chromatogram was stained with rhodamine G. On the right the cephalin compounds (phosphatidylethanolamine and lysophosphatidylethanolamine) are shown by the additional application of a ninhydrin spray. Not indicated are an area above 2 midway between origin and lysolecithin showing a blue fluorescence when wet, and a slightly positive ninhydrin area at the lower edge of lysolecithin in 3′. Time exposure photographs taken under ultraviolet illumination (dry chromatogram, Eastman Panatomic-X film, Wratten filter no. 85, f.12, 5 sec. exposure).

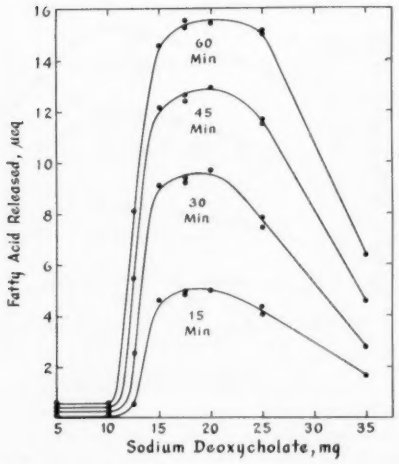

FIG. 2. THE EFFECT OF SODIUM DEOXYCHOLATE ON LECITHINASE A. Separate substrates contained 25 mg (31.1 μM) lecithin and varying amounts of sodium deoxycholate in 5 ml borate buffer, pH 8.4. The enzyme source was diluted 1:16 with the same buffer. After equilibrating separately at 38° C, 1 ml of enzyme was added to each substrate and incubated at 38° C. Fatty acids released were measured at the time intervals shown. Duplicate reaction systems were as plotted.

rum the reaction with duodenal aspirate proceeded only to lysolecithin with the equivalents of fatty acid released equal to the molar decrease in lecithin and increase in lysolecithin as measured by phosphorus analyses. The simpler measure of fatty acids released could then be used in studies of the enzyme.

Selection of buffer. With 1:10 dilutions of the enzyme source in various buffers, 0.05 to 0.1 M, over the pH range 6.0 to 8.1, the greatest liberation of fatty acids occurred in a borate buffer at pH 7.4. The enzymatic activity was slightly less in Tris-(hydroxymethyl)-aminomethane buffer at pH 7.4, and only minimal in phosphate buffer at pH 7.0, the most commonly used buffer in studies of this enzyme.

Effect of sodium deoxycholate. In borate buffer the activity of a 1:25 dilution of enzyme source was 7 per cent of the activity of a 1:10 dilution, instead of the expe ed 40 per cent. It was found that the addition of various bile salts to the system prevented the loss of activity with dilution of the enzyme and that, in appropriate concentration, bile salts had a marked activating effect. Preliminary studies indicated that sodium deoxycholate was the most effective of the bile salts tested. The optimum pH was shifted to 8.4 as will be shown later. Figure 2 illustrates the effect of varying the amount of deoxycholate in the system used. Nonenzymatic reaction in controls was negligible. Twenty and 100 mg amounts of two non-ionic detergents, a polyoxyethylene lauryl ether and a polyoxyethylated nonylphenol, had no activating effect.

Effect of amino acids. Of the amino acids studied, glycine was the most effective in increasing the action of lecithinase A. A marked increase in activity was seen at a 0.01 M concentration, and a point of only slight additional increments in activity was reached at 0.05 to 0.15 M. We elected to use 0.1 M glycine in 0.05 M borate at pH 8.4. Figure 3 compares its activating effect with that of other amino acids. It also shows that varying molarities of borate buffer by itself give identical results.

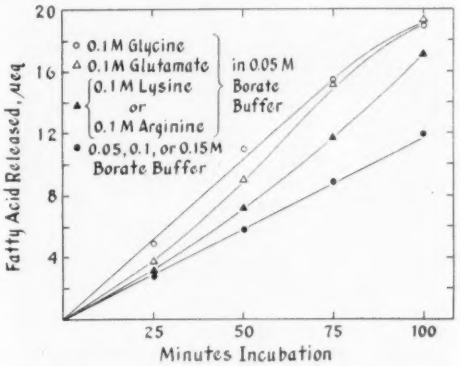

FIG. 3. EFFECT OF AMINO ACIDS ON LECITHINASE A. Substrates contained 25 mg (31.1 μM) lecithin and 20 mg sodium deoxycholate dissolved in either 5 ml of 0.05, 0.10, or 0.15 M borate buffer, pH 8.4, or 5 ml of a 0.1 M solution of glycine, monopotassium glutamate, lysine hydrochloride or arginine hydrochloride in 0.05 M borate buffer, pH 8.4. After equilibrating separately at 38° C, 1.0 ml of enzyme source, diluted 1:32 with 0.05 M borate buffer, pH 8.4, was added to each substrate and incubated at 38° C for the time intervals shown. The results for the three different concentrations of borate buffer and for the lysine and arginine were each so nearly identical that a single curve was drawn through the means of the respective values at the four incubation times.

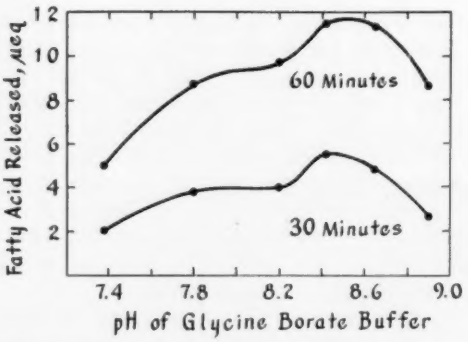

FIG. 4. THE EFFECT OF pH ON THE ACTIVITY OF LECITHINASE A. The reaction system contained 25 mg (31.1 μM) lecithin and 20 mg sodium deoxycholate in 5 ml of 0.1 M glycine in 0.05 M borate buffer at different pH's, and 1 ml of a 1 : 32 aqueous dilution of the enzyme source. Equilibration and incubation at 38° C. No correction was made for the slight effect of deoxycholate on the pH of the system.

Optimum pH. Figure 4 shows that the optimum pH falls between 8.4 and 8.5. Subsequent studies were performed at a pH of 8.45. Studies reported in the literature have been carried out near pH 7.0 (1–6).

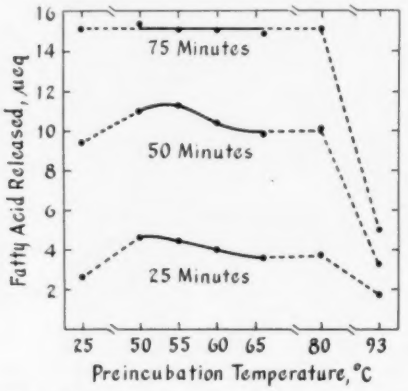

FIG. 5. HEAT STABILITY OF LECITHINASE A. An aliquot of a 1 : 32 dilution of enzyme source in glycine borate buffer was held at room temperature as a control and other aliquots were pre-incubated for 30 minutes at temperatures ranging between 50° and 93° C. After equilibration at 38° C, 1 ml portions of these aliquots were incubated at 38° C with a substrate containing 25 mg (31.1 μM) lecithin and 20 mg sodium deoxycholate in 5 ml glycine borate buffer. Fatty acids released at 25, 50 and 75 minutes were measured. A control incubation with buffer in place of enzyme showed a change of − 0.08, − 0.08, and + 0.06 μEq fatty acids at 25, 50, and 75 minutes, respectively.

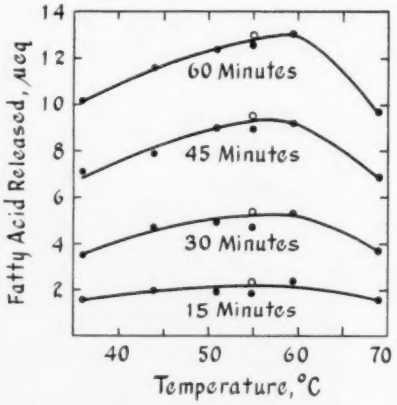

FIG. 6. OPTIMUM INCUBATION TEMPERATURE. Pairs of tubes, one containing 1 ml of enzyme source diluted 1 : 32 and the other 6 ml of substrate, were pre-incubated 30 minutes at temperatures from 36° to 69° C. Then 5 ml of the substrate was added to the enzyme tube and incubated at the pre-incubation temperature. The fatty acids released in 15, 30, 45, and 60 minutes were measured. The effect of pre-incubation for 60 minutes at 55° C is indicated by the open circles. A control of 1 ml of buffer in place of enzyme at 55° C showed a release of 0.18, 0.39, 0.53, and 0.48 μEq fatty acids, respectively, at the time intervals indicated.

Heat stability and incubation temperature. The marked heat stability of lecithinase A reported by others was also found in our assay system as shown in Figure 5. The enzyme was pre-incubated for 30 minutes at different temperatures and then incubated at 38° C. The flattening of the curve at 75 minutes is a reflection of the slowing of the reaction rate when one-half the lecithin is hydrolyzed. The peak of activity, seen at 50° to 60° C for the shorter incubation periods, is further clarified in Figure 6 which presents results of enzyme action with pre-incubation and incubation in the temperature range, 36° to 69° C. The enzyme and substrate in the experiment of Figure 6 were prepared as described in the following paragraph. Wakui and Kawachi (15) recently reported a very similar temperature curve for the enzyme in cobra venom, although they used a different assay system. By way of contrast, lipase is destroyed at 55° C (16).

Optimal incubation system. In subsequent experiments, 0.1 M glycine in 0.05 M borate buffer

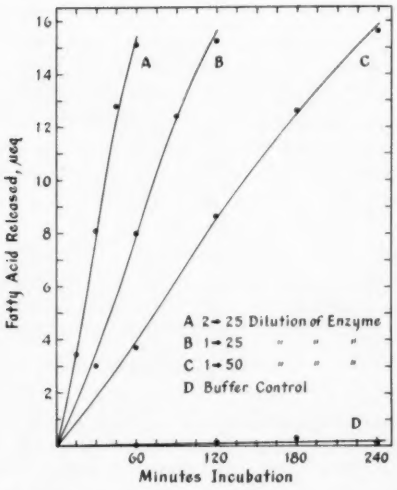

FIG. 7. EFFECT OF DILUTION OF ENZYME ON RATE OF REACTION. Three separate dilutions of the enzyme were incubated in the final system defined in the text. A control of 1 ml of buffer was included.

TABLE I

*The enzymatic conversion of lecithin to lysolecithin**

Sample	Time	Fatty acids released	Lecithin decrease	Lysolecithin increase	Lecithin + lysolecithin
	hrs	*μEq*	*μmoles P*	*μmoles P*	*μmoles P*
A	0				29.9†
	1	5.9	5.0	6.0	30.9
	2	12.0	11.4	10.9	29.4
	18	27.5	26.5	27.0	30.3
B	0				29.4
	18	29.9	28.8	29.8	30.4
C	0				30.7†
	18	29.6	27.7	28.4	30.4
D	0				32.0†
	18	29.6	31.0	29.3	30.3
E	0				30.9†
	18	29.4	29.8	29.5	30.6
Control	0				29.8
	18	0.3	(+0.3)	1.0	31.1
Control	0				30.8
	18	0.6	1.4	0.9	30.3

* Duodenal collections and buffer controls were incubated in the system described. Dilutions were 1:32 for sample A and 1:10 for B through E.

† Average of duplicate chromatography for lecithin and lysolecithin.

at pH 8.45 and room temperature was used to dilute the enzyme and prepare the substrate. The substrate contained 25 mg (31.1 μM) lecithin and 20 mg (48.2 μM) sodium deoxycholate dissolved in 5.0 ml glycine borate buffer. After 1.0 ml enzyme and an adequate volume of substrate were

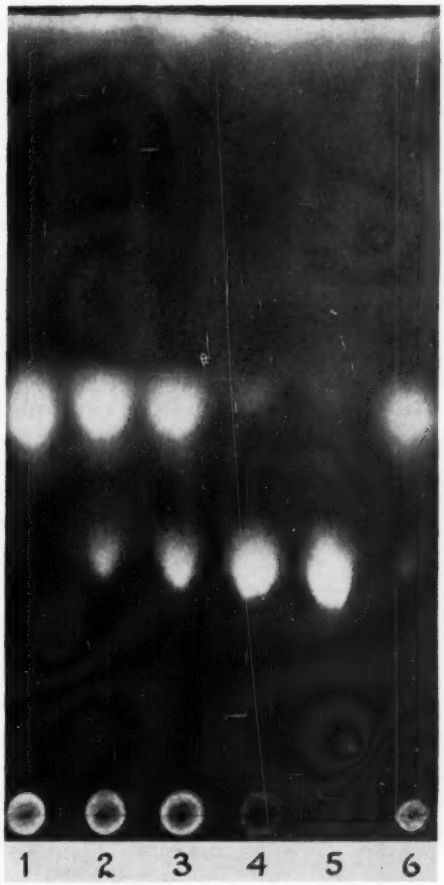

FIG. 8. CHROMATOGRAM OF THE REACTION IN THE INCUBATION SYSTEM. The conversion of lecithin to lysolecithin in Table I, sample A, is shown after chromatography and staining with rhodamine G; 100 μl aliquots of extracts of the 0 time and 1, 2, and 18 hour incubations were applied at origins 1, 2, 3, and 4, respectively. Relatively pure lysolecithin [(β-monostearoyl)-glycerylphosphorylcholine] and lecithin [(dipalmitoleyl)-glycerylphosphorylcholine] were applied at origins 5 and 6, respectively. The deoxycholate of the system appeared as the darker areas above lecithin.

heated separately at 55° C for 30 minutes, 5.0 ml of substrate was added to the enzyme for the incubation at 55° C.

Effect of dilution of the enzyme. As shown in Figure 7, the rate of the reaction was essentially zero order up to the point of 50 per cent utilization of the lecithin. A slight initial lag in the reaction rate is evident.

Test of the system. Five duodenal collections from different individuals were incubated in the optimal system. The release of fatty acids and the conversion of lecithin to lysolecithin were measured. The data in Table I show that the two methods of measuring activity check closely, and that even at the prolonged period of 18 hours the reaction proceeds only to lysolecithin as evidenced by the recovery of the original lecithin phosphorus. A chromatogram illustrating the reaction in sample A, Table I is shown in Figure 8.

Effect of reaction products. The effect of the reaction products of the system is shown in Figure 9. A slight activating effect of lysolecithin was observed. Oleic acid was used as a representative fatty acid reaction product. It was inhibitory at a concentration which is present when 50 per cent of the lecithin in the system has been hydrolyzed.

Inhibitory factors. Suppression of enzymatic activity by various substances is illustrated in Figure 10. The treatment of enzyme and substrate with diethyl ether was prompted by the observation that inhibition occurred when lecithin was incorporated into the substrate by evaporation of a redistilled peroxide free ether solution of lecithin overlying the buffer. As can be seen in Figure 10, the depressant effect of ether is upon the substrate rather than the enzyme. Both oxalate and calcium have depressant effects. As the concentration of calcium decreased the depressant effect decreased. We have observed the effect of magnesium to be similar to that of calcium. The effect of ethylenediamine tetraacetate was not abolished by first saturating it with calcium, suggesting that its depressant effect was not related to its chelating property. Dialysis of duodenal contents did not reduce activity of the enzyme.

Using the diethyl ether method of Hanahan, without added calcium, the reaction proceeds with 1 ml of undiluted enzyme source to 50 per cent conversion of 31 μmoles of lecithin in 24 hours.

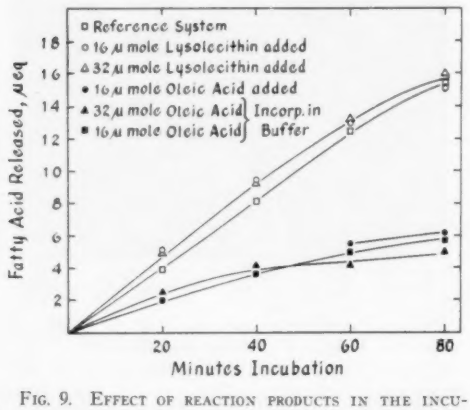

FIG. 9. EFFECT OF REACTION PRODUCTS IN THE INCUBATION SYSTEM. The substrate as defined in the text was modified for three incubations to incorporate 16 or 32 μmoles of lysolecithin or 16 μmoles of oleic acid by addition to the lecithin prior to evaporation of the solvent and incorporation into the substrate. For two incubations oleic acid was added to the glycine and boric acid prior to adjusting to pH 8.45, giving 16 or 32 μmoles of oleic acid in the substrate.

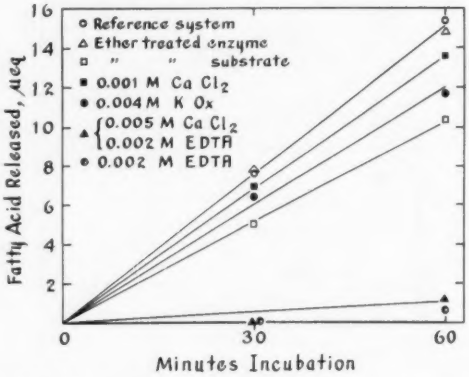

FIG. 10. INHIBITION OF LECITHINASE A. Separate standard substrates as defined were modified to include at pH 8.45 a concentration of: *1*) 0.001 M calcium chloride, *2*) 0.004 M potassium oxalate, *3*) 0.002 M EDTA (ethylenediamine tetraacetate), or *4*) both 0.005 M calcium and 0.002 M EDTA. In two additional incubations enzyme and substrate were used from which ether had been evaporated prior to pre-incubation heating. In one, 1 ml of Mallinckrodt anhydrous analytical ether was evaporated from each milliliter of enzyme. In the other, 1.67 ml of the same ether was evaporated from each milliliter of substrate.

DISCUSSION

The use of bile salts and amino acids in the system proposed was prompted by their use to en-

hance lipase activity (16). The formation of choleic acid complexes by deoxycholate (16) and the binding of deoxycholate by calcium must be considered in interpretation of inhibition of the system. Such effects may be due to reducing available deoxycholate below the optimal requirement. Calcium has been shown to be inhibitory for lecithinase A from rat intestinal mucosa (5) but is required for the enzyme in venom (4) and in ox pancreas (6). In our assay system with duodenal drainage, calcium is inhibitory. The concentration of calcium contributed by normal duodenal drainage amounts to 0.00004 M, approximately. The quantitative requirement for deoxycholate in the system may be less if it is prepared by means other than precipitation from ether, as it is known that deoxycholate binds ether tenaciously (14). We have also observed that sodium taurocholate and glycocholate, reprecipitated as described, show the same effect on the release of fatty acids as an equimolar amount of deoxycholate.

The enzyme from rat intestinal mucosa is activated by oleic acid, inhibited by cholic acid and is heat labile (5). The marked stability to heat and inhibition by reaction products shown by the enzyme from duodenal drainage is in agreement with studies reported for the enzyme from snake venom, ox pancreas or pancreatin.

The presence and action of the enzyme explains the observation of Borgström (7) that lecithin is converted to lysolecithin in the upper intestine.

SUMMARY

A lecithinase A was demonstrated in the duodenal contents of man. The release of fatty acids from lecithin was measured titrimetrically. The decrease in lecithin and equivalent increase in lysolecithin were determined independently by quantitative paper chromatography. The action of the enzyme was markedly increased in the presence of sodium deoxycholate and moderately increased by glycine, glutamic acid, arginine or lysine. The optimum medium used for the hydrolysis was a glycine-borate buffer at pH 8.5, and the optimum temperature was 55° C. Heating in this medium at 80° C for 30 minutes did not inactivate the enzyme. The enzyme was partially inhibited by calcium, magnesium, oxalate, oleic acid, and diethyl ether, and nearly completely inhibited by ethylenediamine tetraacetic acid.

ACKNOWLEDGMENT

We are indebted to Dr. U. S. Seal for helpful criticisms and to Dr. W. J. Henke for the duodenal aspirations.

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STUDIES ON THE RENAL CONCENTRATING MECHANISM. V. EFFECT OF DIURETIC AGENTS *

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Evidence for the site of action of diuretic drugs in man can be derived from observations of their effect on different tubular functions. Previous studies have shown that mercurial diuretic agents have little influence on water diuresis or antidiuresis (1–5) and hydrogen ion secretion (6, 7). These observations have been taken to indicate that the predominant action of these mercurial agents is to decrease sodium reabsorption in the proximal tubule. A proximal site of action has also been suggested by stop flow analysis of tubular function during combined mercurial and mannitol diuresis (8–10). The similarity between osmotic and mercurial diuresis has been emphasized (5, 8, 11, 12) but these states have not been compared directly in man. The present study concerns the effects of diuretic drugs on renal concentrating ability in healthy hydropenic young men. The urine was found to be less concentrated during mercurial diuresis when compared with mannitol diuresis at similar urine flows. This difference was greatest when mercurial action was potentiated by the prior administration of ammonium chloride. The data provide evidence that mercurial diuretic agents can act on those portions of the nephron involved in the renal concentrating process, presumably beyond the proximal tubule. Acetazolamide and chlorothiazide, drugs which probably act both distally and proximally (8, 13–15) were also studied. The former was found to decrease concentrating ability, while the latter had little effect.

METHODS

Experimental procedure. The subjects were 6 paid volunteer medical students, ages 25 to 28 years, who were selected by a "bladder emptying test" for their ability to void small volumes of urine consistently (16). The experiments were begun after 18 hours of water deprivation, at 7 a.m., except in Subject A.G., in whom experiments were begun at 11 a.m. Infusions containing the diuretic agents were administered after 2 to 3 preliminary periods, in which osmotic urine/plasma ratio ranged from 3.0 to 4.6, and urine flow was less than 0.6 ml per minute. Urine samples were collected at intervals of 30 minutes and blood samples every 2 hours.

The following experiments were performed in a random sequence for each subject, at intervals of at least 1 week between studies. In all experiments 200 mU of vasopressin [1] was administered initially. Vasopressin was then added to each infusion to provide 200 mU per hour, except in the repeat experiments with mercaptomerin.

1. Mannitol infusion (all subjects); 5 per cent mannitol in 0.5 per cent NaCl solution was infused at a rate of 10 ml per minute for 5 hours.

2. Urea infusion (3 subjects); 30 per cent urea was infused at 1 to 3 ml per minute for 4 hours.

3. Isotonic sodium chloride infusion (3 subjects); 0.9 per cent NaCl solution was infused at 30 to 40 ml per minute for 1 hour, then at 10 ml per minute for the next 3 hours, giving a total volume of about 4 L.

4. Mercaptomerin (all subjects); a solution of mercaptomerin containing 40 mg Hg in 50 ml of isotonic sodium chloride was injected intravenously, followed by an

TABLE I

*Mannitol loading "reference" experiment**

Subject	No. of periods	a	b	R
M.G.	8	0.0874	0.3886	0.9929
P.M.	8	0.1222	0.2839	0.9928
A.M.	7	0.0910	0.3817	0.9961
J.P.	8	0.0940	0.3031	0.9953
J.S.	7	0.1041	0.2442	0.9931
A.G.	8	0.1168	0.3202	0.9948

* The equation, $\frac{1}{U/P - 1} = aV + b$, for the mannitol loading "reference" experiment for each subject, is presented in terms of the regression constants (a, b) and the correlation coefficient (R).

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[1] A single lot of Pitressin (Parke, Davis) was used which had been shown to cause maximal antidiuresis in hydrated normal man in doses of 25 to 50 mU per hour.

TABLE II

*Summary of individual diuresis experiments**

Experiment	Subject	Range			Mean		
		V	U/P		ΔU/P	$\Delta T^c_{H_2O}$	C_{creat}
		ml/min/1.73 m²					*ml/min/1.73 m²*
Mannitol	M.G.	1.39–6.35	2.07–3.05				140
	P.M.	1.91–7.37	1.85–3.01				145
	A.M.	1.41–7.43	1.95–3.01				105
	J.P.	1.31–4.74	2.35–3.44				128
	J.S.	0.89–5.36	2.27–3.91				150
	A.G.	1.60–7.34	1.86–3.06				131
				Mean			133
Urea	M.G.	1.37–4.17	2.30–3.02		−0.06	−0.19	111
	P.M.	1.10–4.56	2.21–3.01		−0.16	−0.31	166
	A.M.	1.52–4.28	2.31–3.30		0.06	0.10	132
				Mean	−0.05	−0.13	136
Saline	M.G.	1.06–2.58	2.58–3.03		−0.04	−0.06	180
	P.M.	0.78–2.33	2.49–3.42		−0.24	−0.39	145
	J.P.	0.96–2.47	2.90–3.72		0.08	0.10	193
				Mean	−0.07	−0.12	173
Mercaptomerin	M.G.	2.77–6.98	1.54–2.21		−0.44	−1.85	142
	P.M.†	2.40–6.92	1.46–1.99		−0.55	−2.34	130
	A.M.	1.20–4.21	1.68–2.85		−0.53	−1.63	122
	J.P.	1.28–8.97	1.42–2.45		−0.61	−3.16	125
	J.S.	1.72–5.27	1.64–2.40		−0.71	−2.76	124
	A.G.	3.87–9.79	1.30–1.73		−0.41	−2.68	132
				Mean	−0.54	−2.40	129
Meralluride	J.P.	1.06–6.38	1.49–3.00		−0.68	−2.36	122
	J.S.	1.52–6.74	1.64–2.18		−0.71	−2.49	135
	A.G., 1	1.61–3.29	2.49–3.15		0.07	0.15	154
	A.G., 2	1.86–4.18	2.17–2.74		−0.10	−0.32	150
				Mean‡	−0.69	−2.42	128
NH_4Cl + mercaptomerin	M.G.	1.56–8.99	1.28–2.16		−0.78	−4.08	143
	J.P.	4.47–16.1	1.19–1.33		−0.64	−5.45	132
	J.S.	1.41–11.9	1.16–2.22		−0.85	−5.27	103
				Mean	−0.76	−4.94	126
Chlorothiazide	M.G.	2.52–3.34	2.27–2.69		−0.09	−0.27	139
	P.M.	2.47–5.66	1.81–2.44		−0.15	−0.60	153
	A.M.	2.36–3.26	2.33–2.76		−0.02	−0.05	114
	J.P.	1.66–3.87	2.36–2.86		−0.15	−0.36	131
	J.S.	2.02–2.70	2.48–2.81		−0.43	−1.05	117
	A.G.	1.91–3.27	2.04–2.77		−0.13	−0.37	106
				Mean	−0.16	−0.45	127
Acetazolamide	J.P.	1.56–2.42	2.32–2.74		−0.44	−1.01	125
	J.S.	1.56–2.82	2.10–2.32		−1.02	−1.89	142
	A.G.	1.14–3.58	2.14–2.97		−0.23	−0.63	166
	P.M.	1.90–4.08	1.91–2.50		−0.46	−1.19	134
				Mean	−0.54	−1.18	142

* Including range of urine flow (V), osmotic U/P ratio (U/P), mean creatinine clearance (C_{creat}), and deviation of osmotic U/P ratio (ΔU/P) and net water reabsorption ($\Delta T^c_{H_2O}$) from the values predicted from the mannitol loading "reference" experiment.

† Three 60 minute periods only.

‡ Mean of Subjects J.P. and J.S. only.

infusion containing 40 mg Hg in 240 ml of isotonic NaCl solution at a rate of 1.0 ml per minute. This experiment was repeated in Subjects J.S. and J.P. with the addition of 1,000 mU per hour of vasopressin.

5. Meralluride (3 subjects); the procedure and dose were identical with that for mercaptomerin. The experiment was repeated in Subject A.G.

6. Ammonium chloride and mercaptomerin (3 subjects); the mercaptomerin experiment was repeated after the subjects had received 9 to 12 g ammonium chloride orally per day for 3 days.

7. Chlorothiazide (all subjects); 0.5 g of chlorothiazide dissolved in 50 ml isotonic NaCl solution was injected intravenously followed by an infusion of 0.5 g chloro-

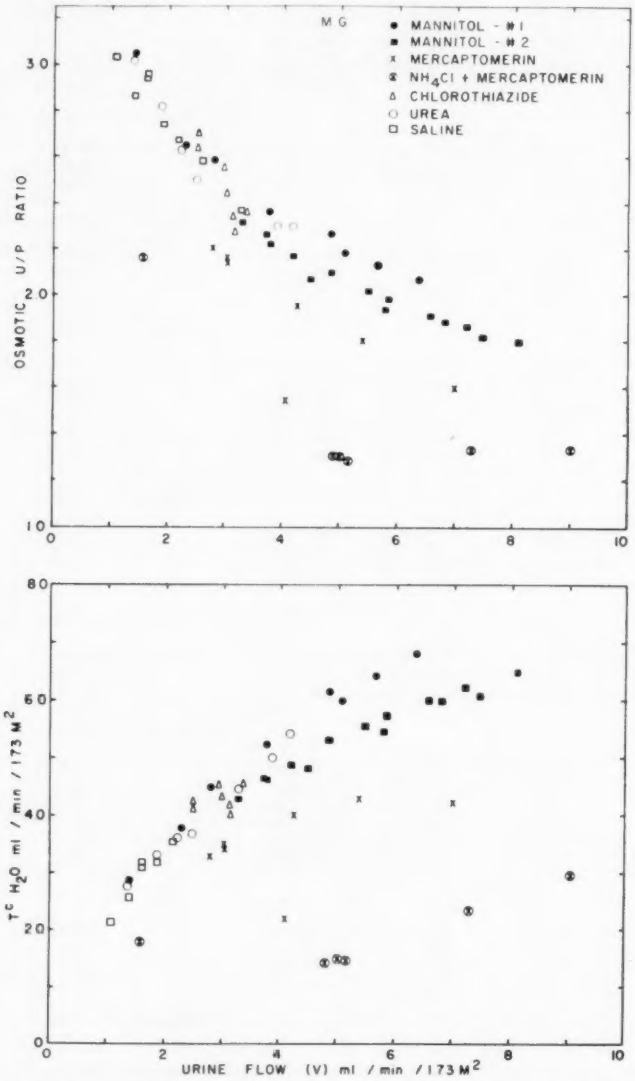

FIG. 1. CONCENTRATING RESPONSE TO VARIOUS DIURETIC AGENTS IN SUBJECT M.G. Mannitol no. 1 indicates the "reference" mannitol loading experiment for this subject. Mannitol no. 2 is a study done one year previously.

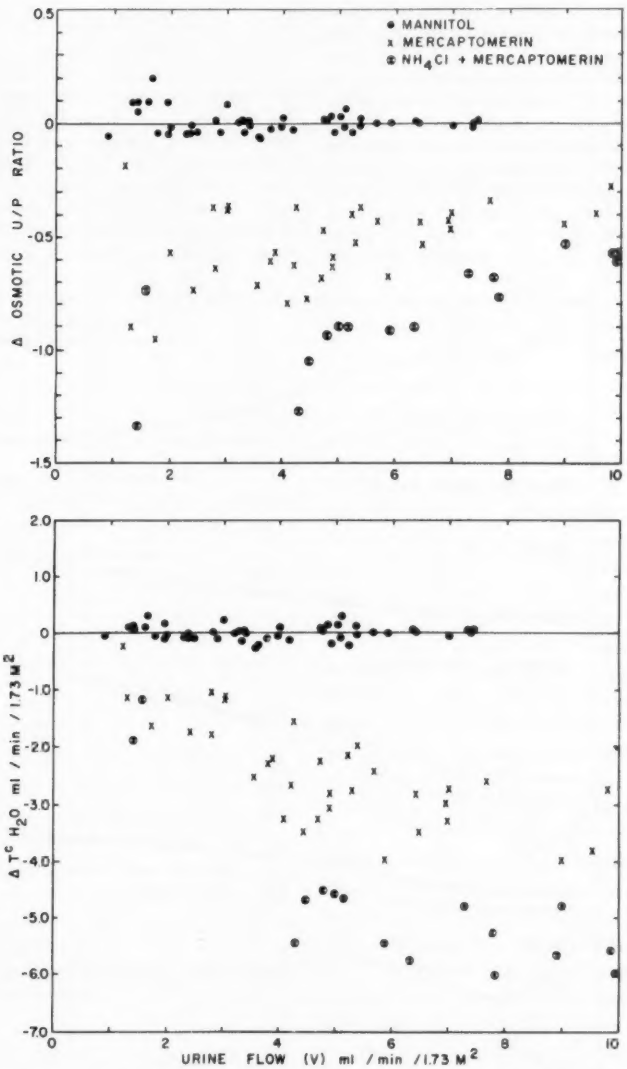

FIG. 2. DEVIATION OF Δ OSMOTIC U/P RATIO AND NET WATER REABSORPTION ($\Delta T^c_{H_2O}$) FROM VALUES PREDICTED BY THE MANNITOL "REFERENCE" EQUATION (SEE TEXT). Data include periods for mannitol, mercaptomerin, and ammonium chloride-potentiated mercaptomerin diuresis in six subjects.

thiazide in 240 ml of isotonic NaCl solution at a rate of 1.0 ml per minute.

8. Acetazolamide (4 subjects); the procedure and dose were identical with that for chlorothiazide.

9. Mannitol infusion and mercaptomerin (3 subjects); 10 per cent mannitol was infused at 20 ml per minute until urine flow was 10 to 15 ml per minute for 3 to 4 periods. Mercaptomerin (80 mg Hg) was then given intravenously and the mannitol infusion continued at 10 ml per minute for 3.5 hours.

Chemical methods. Chemical methods were those previously described (16). In addition, chloride was determined by the method of Schales and Schales (17).

Calculations. Osmotic urine/plasma (U/P) ratio,

osmolal clearance (C_{osm}), and net water reabsorption ($T^c_{H_2O}$) were calculated as described previously (16, 18, 19). All data were corrected to 1.73 m^2 of body surface area. The relationship between concentrating response and urine flow (V) during osmotic diuresis, when urine flow is less than 20 ml per minute, can be evaluated in terms of an empirical formula described in the preceding paper of this series (19). In the present study, the mannitol loading experiments on each subject were chosen as the "reference" experiment for comparison of the renal concentrating response with other types of diuresis. The regression constants and the correlation coefficient for the hyperbolic equation, $\frac{1}{U/P-1} = aV + b$, where U/P represents the osmotic U/P ratio, V is urine flow, and a and b are constants, were calculated for each mannitol loading "reference" experiment by the method of least squares [(20) Table I]. Using this equation, it was possible to calculate predicted values for osmotic U/P ratio and $T^c_{H_2O}$, [$T^c_{H_2O} = (U/P - 1) \times V$] for any given rate of urine flow. Differences in concentrating response between the mannitol experiment and other forms of diuresis in each subject could then be quantitated by comparing observed osmotic U/P ratio and $T^c_{H_2O}$ values

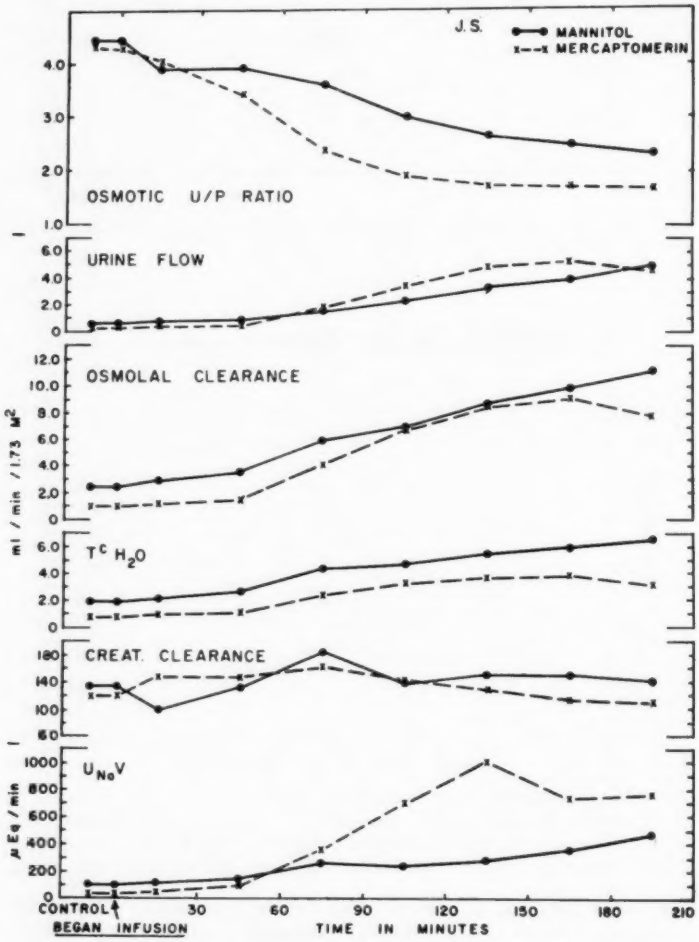

FIG. 3. COMPARISON OF INDIVIDUAL EXPERIMENTS WITH MANNITOL LOADING AND MERCAPTOMERIN DIURESIS IN SUBJECT J.S. Osmotic U/P ratio, urine flow, osmolal clearance, net water reabsorption ($T^c_{H_2O}$), creatinine clearance, and sodium excretion ($U_{Na}V$) are given for individual periods.

TABLE III

*Effect of mercaptomerin during hypertonic mannitol loading**

Subject	Time	V	U/P	$T^c_{H_2O}$	$U_{Na}V$	$\frac{U_{Na}}{U_{osm}} \times 100$
	min	*ml/min/1.73 m²*		*ml/min/1.73 m²*	*μEq/min/1.73 m²*	
J.P.	−40–0	14.0	1.50	7.0	577	9
	70–110	19.2	1.32	6.2	1,127	15
	150–180	20.6	1.34	6.9	1,144	14
J.S.	−40–0	11.3	1.57	6.4	370	7
	70–110	12.8	1.46	5.8	705	12
	150–170	17.4	1.36	6.2	1,234	17
A.G.	−45–0	10.8	1.48	5.2	515	11
	75–120	21.7	1.18	3.8	1,741	23
	150–195	21.3	1.26	5.6	1,515	19

* Mean urine flow (V), osmotic U/P ratio (U/P), net water reabsorption ($T^c_{H_2O}$), sodium excretion ($U_{Na}V$) and percentage of urine osmolality accounted for by sodium $\left(\frac{U_{Na}}{U_{osm}} \times 100\right)$ are given for periods before, 1 to 2 hours after, and 2.5 to 3 hours after mercaptomerin administration.

with those predicted for the same rate of urine flow by the equation for the mannitol "reference" experiment. The differences between observed and predicted values are termed Δ U/P and $\Delta T^c_{H_2O}$. In 3 subjects, M.G., P.M. and A.M., data from previous mannitol loading experiments (19) over the range of 1 to 20 ml per minute urine flow showed good agreement with the mannitol "reference" experiment, so that calculated Δ U/P values for individual collection periods were always between + 0.15 and − 0.30.

RESULTS

The renal concentrating response during different types of diuresis is illustrated for one subject in Figure 1. The mean values for Δ U/P and $\Delta T^c_{H_2O}$ for each experiment are given in Table II. The concentrating response to urea and saline diuresis showed no consistent deviation from that observed with mannitol loading, although in individual periods Δ U/P ranged from + 0.38 to − 0.50 and $\Delta T^c_{H_2O}$ from + 0.58 to − 0.73 ml per minute.

A consistently lower osmotic U/P ratio and smaller $T^c_{H_2O}$ was observed during mercaptomerin diuresis as compared with osmotic diuresis at similar urine flows (Figure 2, Table II). Mean Δ U/P for all six subjects was − 0.54 (individual periods ranged from − 0.19 to − 0.96) and $\Delta T^c_{H_2O}$ was − 2.40 ml per minute (individual periods ranged from − 0.24 to − 4.00 ml per minute). The contrast between mannitol loading and mercaptomerin experiments in one subject, J.S., is shown in Figure 3. Depression of osmotic U/P ratio appeared early in mercurial diuresis and preceded the peak natriuretic response. An even greater change in concentrating response is observed during mercaptomerin diuresis which has been potentiated by prior administration of ammonium chloride (Table II). Although larger peak urine flows were also achieved, greater deviations in the concentrating response were seen even at lower rates of urine flow (Figures 1, 2). When the dose of vasopressin was increased to 1,000 mU per hour in repeat mercaptomerin experiments on J.S. and J.P., the depression of concentrating ability was as great as that observed in the original mercaptomerin experiment (mean Δ U/P values were − 0.65 and − 0.69, respectively).

The concentrating response during meralluride diuresis was the same as that observed for mercaptomerin in Subjects J.P. and J.S. In Subject A.G. there was no impairment of concentrating ability in the first experiment in which meralluride caused only a small increase in urine flow and sodium excretion. In the second meralluride experiment, Subject A.G. showed a greater diuresis but only a slight decrease in concentrating ability.

When mercaptomerin was administered during a hypertonic mannitol infusion there was a small decrease in $T^c_{H_2O}$ despite a further increase in urine flow; 2.5 to 3 hours after mercaptomerin administration $T^c_{H_2O}$ had returned toward control values, although urine flow and sodium excretion remained high (Table III).

Chlorothiazide and acetazolamide yielded considerably smaller increases in urine flow than did

TABLE IV

*Effect of diuretic agents on urinary composition**

Diuretic agent	No. of experiments	V	$U_{osm}V$	$U_{Na}V$	U_KV	$U_{Cl}V$	$U_{urea}V$	$\frac{U_{Na}}{U_{osm}} \times 100$	$\frac{U_K}{U_{osm}} \times 100$
		ml/min	*μOsm/min*		*μEq/min*		*μmoles/min*		
Mannitol	6	5.98	3,612	470	80	435	333	13.0	2.2
Isotonic NaCl	3	2.36	1,838	582	116	614	393	31.7	6.3
Urea	3	4.09	2,898	420	103	380	1,925	14.5	3.6
Mercaptomerin	6	6.59	2,903	1,155	68	1,170	317	39.8	2.3
NH_4Cl + mercaptomerin	3	11.0	3,812	1,360	112	1,581	266	35.6	2.8
Meralluride†	2	6.18	2,882	1,192	34	1,035	289	41.4	1.2
Chlorothiazide	6	3.35	2,228	777	187	576	342	34.5	8.4
Acetazolamide	4	2.82	1,776	568	230	244	320	31.6	13.1

* Urine flow (V), total solute ($U_{osm}V$), sodium ($U_{Na}V$), potassium (U_KV), chloride ($U_{Cl}V$), and urea ($U_{urea}V$) excretion, and percentage of total solutes accounted for by Na $\left(\frac{U_{Na}}{U_{osm}} \times 100\right)$ and K $\left(\frac{U_K}{U_{osm}} \times 100\right)$ are given for the maximal hourly urine flow.

† Subjects J.P. and J.S. only.

mercaptomerin. During chlorothiazide diuresis concentrating ability was only slightly less than that observed with mannitol loading, except in Subject J.S. who showed a definite decrease. Acetazolamide caused a distinct decrease in concentrating ability, and in Subject J.S. this decrease was relatively greater than that observed with mercaptomerin.

Endogenous creatinine clearance values were little affected by any of the agents studied, except for an increase with saline loading. Transient depressions of creatinine clearance were not encountered with any of the agents used in these experiments.

The patterns of urinary electrolyte composition observed during the peak response to the various agents are shown in Table IV. Mercurial diuretic agents primarily increased sodium and chloride excretion, while chlorothiazide and acetazolamide increased potassium as well as sodium excretion and caused less increase in chloride excretion. The resultant increase in undetermined anion was presumably due to increased bicarbonate excretion. This difference was greatest with acetazolamide diuresis. There were no consistent changes in plasma composition with any of the agents used except for a decrease in plasma bicarbonate concentration to 17 mmoles per L after three days of ammonium chloride administration.

DISCUSSION

The urine is less concentrated during mercurial diuresis than it is during osmotic diuresis at comparable urine flows in the same subjects. This apparent impairment of the ability of the kidney to elaborate a concentrated urine could be due to inhibition of antidiuretic hormone, to a direct effect on water transport, or to inhibition of a moiety of sodium reabsorption which is essential to the concentrating process. Inhibition of antidiuretic hormone could decrease concentrating ability by altering water reabsorption (*21*) or by altering sodium reabsorption in the loop of Henle (*22*) if antidiuretic hormone increases sodium reabsorption at this site as it does in the short-circuited toad bladder (*23*). A recent study has suggested that antidiuretic hormone might act on the renal tubule by forming disulfide linkages with proteins in the cell membrane (*24*). Since mercurial diuretic compounds are known to bind available sulfhydryl groups (*25*) these agents might compete for available binding sites. Competitive inhibition of antidiuretic hormone action could not be demonstrated in the present study, however, since a fivefold increase in the dose of exogenous vasopressin did not alter the mercurial effect on concentrating ability. Mercurial diuretic agents might act on concentrating ability by directly inhibiting water transport. Since net water reabsorption at the concentrating site is probably passive in response to an osmotic gradient (*21, 22, 26*), such an inhibition should then be due to decreased membrane permeability. However, Garby and Linderholm (*27*) have shown that mersalyl has no effect on the permeability of frog skin to heavy water, but it does cause a de-

crease in active sodium transport. Could impairment of concentrating ability be ascribed to the direct action of organomercurial agents on sodium transport in the renal tubules? It has been postulated on the basis of stop flow analysis that mercurial diuretic agents act by inhibiting active (9) or passive (10) sodium reabsorption in the proximal tubule. Such an effect would not explain impairment of concentrating ability relative to that observed during osmotic diuresis since the latter also decreases proximal sodium reabsorption (28).

Recent evidence indicates that the renal concentrating process operates by means of a countercurrent multiplier system which is initiated by active sodium transport in the loop of Henle (21, 22, 26, 29, 30). This produces a hypertonic renal medullary interstitial fluid, which results in passive reabsorption of water from the collecting ducts. If this hypothesis is correct, it then appears likely that mercurial diuretic agents impair concentrating ability by inhibiting sodium transport in the loop of Henle. The localization of mercurial diuretic action to this site is further suggested by histochemical data which show that the number of available sulfhydryl binding sites in the ascending portion of the loop of Henle is decreased after the administration of mersalyl (25).

The greater impairment of concentrating ability observed when mercurial diuretics were given during ammonium chloride acidosis is not unexpected if acidosis potentiates mercurial inhibition of sodium transport at the concentrating site. Ammonium chloride acidosis itself did not impair renal concentrating ability in preliminary periods in these subjects or in three additional subjects studied before and after the administration of ammonium chloride for three days. Moreover, intravenous acid loading in hydropenic dogs does not appear to affect renal concentrating ability (31, 32).

Previous studies assigning mercurial action to the proximal tubule have cited the apparent lack of effect of these drugs on net water reabsorption during antidiuresis (3, 5) and on free water clearance during maximal water diuresis (1, 2). In a more recent study, free water clearance was found to increase during combined mercurial and water diuresis (33) but this could still be consistent with a proximal site of action. Although direct comparison of osmotic and mercurial diuresis was not made, in one of the available studies in man (5) concentrating ability may have been depressed during mercurial diuresis, since values of $T^c_{H_2O}$ were generally less than 4 ml per minute when urine flow was 15 ml per minute. In hydropenic dogs, Brodsky and Graubarth (11) found no difference in the relationship between osmotic load and urine flow when they compared meralluride and mannitol diuresis. The effects of meralluride and mercaptomerin on the concentrating mechanism were different in one subject of the present study, but identical in two others.

In the present study there was little alteration in the rate of net water reabsorption when mercurial diuresis was superimposed on a mannitol diuresis at high urine flows. Interpretation of these data is difficult since $T^c_{H_2O}$ can vary with different types of osmotic diuresis at high urine flows (19). If, as Malvin and Wilde (34) have recently suggested, the countercurrent gradient is "washed out" during massive osmotic diuresis then, under these circumstances, the action of inhibitory drugs on the concentrating mechanism could be less apparent.

Impairment of concentrating ability during acetazolamide diuresis suggests that carbonic anhydrase inhibition may affect sodium transport in the loop of Henle. The increased bicarbonate ion in the tubule does not appear to be a factor since sodium bicarbonate infusions in dogs (30) and in one of our subjects (unpublished observations) did not impair concentrating ability. Chlorothiazide, which in addition to inhibiting carbonic anhydrase to a lesser extent appears to act chiefly on sodium chloride reabsorption (8, 13, 35), caused little impairment of concentrating ability. Others have found no impairment of net water reabsorption during mannitol diuresis in subjects given chlorothiazide (33, 36). These results with chlorothiazide would indicate that the difference between mannitol and mercurial diuresis is not due to the fact that sodium chloride is the predominant urinary solute during drug-induced diuresis. Actually, sodium chloride diuresis produces a somewhat more concentrated urine than does mannitol diuresis in hydropenic rats (22).

Impairment of concentrating ability during mercurial diuresis has been quite transient in the subjects of the present study. Further studies are

required to determine whether this effect is similarly transient in patients requiring diuretic therapy. The fact that the urine is less concentrated during mercurial diuresis may actually represent an advantage to the patient with edema, who must excrete an excess of isotonic extracellular fluid, in the presence of maximal antidiuretic hormone action.

SUMMARY

The effect of osmotic (mannitol, urea, saline), mercurial, ammonium chloride-potentiated mercurial, chlorothiazide, and acetazolamide diuresis on renal concentrating ability was determined in six hydropenic young men. There was no consistent difference between the response of the concentrating mechanism to mannitol, urea, and saline diuresis at low rates of urine flow. A marked decrease in concentrating ability was seen during mercaptomerin diuresis as compared with mannitol diuresis at the same urine flows. Meralluride diuresis was associated with a similar decrease in two of three subjects studied. When mercaptomerin action was potentiated by prior administration of ammonium chloride, there was greater apparent impairment of concentrating ability than with mercaptomerin alone. This impairment indicates that mercurial diuretic agents act at the renal concentrating site as well as on the proximal tubule. Acetazolamide was also found to cause a decrease in renal concentrating ability while chlorothiazide had little effect.

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THE PLASMA DISAPPEARANCE OF INTRAVENOUSLY ADMINISTERED COBALT^{58} VITAMIN B_{12} *

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The combination of low serum content and low specific activity tracer material has presented a difficult technical barrier to the study of the plasma disappearance of tracer amounts of vitamin B_{12}. Three groups (1–3) have studied the rate of plasma disappearance of intravenous doses ranging from 0.5 to 4.0 μg. These doses were not and could not be considered to be of tracer magnitude since even 0.5 μg may equal the entire plasma content. Estren, Brody and Wasserman (3) in their review gave 2×10^{-4} to 9×10^{-4} μg per ml as the normal range of serum vitamin B_{12}. Thus, persons with plasma volumes of 3,000 ml would have a total plasma content of between 0.6 and 3 μg. All three groups referred to above found a rapid plasma loss of the labeled B_{12} in normal subjects. Patients with chronic myelogenous leukemia (1–3) retained more of the vitamin in the plasma, and it was assumed that the greater plasma-binding capacity of these patients prevented the escape of the vitamin.

If plasma binding determines the rate of loss from the circulation, the rapid loss described in normal subjects (1–3) may have been a consequence of the relatively large amounts of labeled material used. The measurement of the disappearance of truly tracer amounts of vitamin B_{12} appeared necessary for a proper understanding of the process.

The term "binding" is used here according to current concepts of vitamin B_{12} plasma binding. A detailed discussion is beyond the scope of this paper, but the many approaches which give similar but not identical values can be summarized under three categories: *1*) the use of dialysis or a related process for the separation of free and bound vitamin; *2*) the use of charcoal extraction for the same purpose; *3*) making use of the unavailability of bound vitamin to certain microorganisms until the serum is heated. Estren and associates (3) stated the unsaturated serum binding capacity to be on the order of 7×10^{-4} μg per ml. This amount can be bound in addition to that already present and in a bound form. A person with a plasma volume of 3,000 ml would then have a total unsaturated binding capacity on the order of 2 μg.

An important contribution to the problem of binding was recently made by Miller and Sullivan (4) who found a seromucoid with specific mobility on electrophoresis to be the binding site of native vitamin B_{12}. However, added B_{12} could be bound to other sites firmly enough to resist removal by dialysis, and it appears that binding as measured by current *in vitro* techniques may measure more than that vitamin attached to the native binding site. Too little is known about the relationships between the plasma binding of vitamin B_{12} and vitamin B_{12} metabolism to permit the assumption that binding at a site different from that of the native B_{12} does not have biological significance. This recently described more restricted concept of binding is presented here mainly to emphasize that while *in vitro* binding of B_{12} can be measured easily and precisely by charcoal extraction or dialysis techniques, one must be cautious in relating it to *in vivo* events until it is better understood. For the present study, bound vitamin was considered to be that vitamin which could not be removed by charcoal extraction.

Fortunately, a high specific activity cobalt^{58} vitamin B_{12} ($Co^{58}B_{12}$) has recently become available.[1] This development and the adaptation of more sensitive counting techniques made it possible to use very small amounts of labeled material in the present study. The smaller doses used were on the order of 1/50 to 1/250 of the entire

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[1] Supplied through the kindness of Dr. Nathaniel S. Ritter, Merck, Sharp and Dohme Research Laboratories, Rahway, N. J.

plasma content and could be completely bound by 30 to 40 ml of plasma and serum prior to injection. The purpose of the study was to describe the plasma disappearance of such a dose and to study factors influencing it.

MATERIALS AND METHODS

Subjects. The subjects were hospitalized patients past age 50. With the exception of the two patients who received vitamin B_{12} mixed with large volumes of plasma, all of the control subjects had recovered from an acute illness. Patients with liver disease, alcoholism, malnutrition, gastrointestinal disease, neoplasms, leukemia, renal disease, diabetes, or endocrine disorders were excluded. All of the control subjects had normal serum vitamin B_{12} levels and normal serum binding capacities.

Radioactive vitamin B_{12}. All plasma and urine studies were made with two lots of $Co^{58}B_{12}$ of initial specific activities of 8.1 and 6.1 μc per μg. The body-counting studies were done with one lot of 9.7 μc per μg. The majority of the studies were performed within one month of receipt of shipment, a very few after two months. The vitamin B_{12} activity of each lot was checked by *Euglena gracilis* assay. One lot was checked for free Co^{58} by half-saturating a plasma with a known binding capacity and removing free Co^{58} by dialysis, and none was detected.

Vitamin B_{12} assay. The *E. gracilis* Z strain method of Hutner, Bach and Ross (5) was used throughout. Serum B_{12} binding capacity was measured by the charcoal extraction method of Miller (6). Charcoal extraction was also used to determine the state of B_{12} in mixtures of $Co^{58}B_{12}$ and serum.

Sampling procedure. Thirty ml venous samples were taken at intervals and all measurements were performed on serum. Start and stop times of each sample were recorded, and the half-times were used in the calculations.

Counting procedures. The serum samples of 10 to 15 ml were counted in flat-bottomed vials fitting snugly in a large diameter (hole, 1 inch) well crystal. Urines were counted in volumes of up to 600 ml in a beaker so designed that a conventional sized well crystal protruded into the beaker from below. Both systems were shielded with 2 inches of lead. All counting was done with a Baird-Atomic model 513 scintillation spectrometer set to include only the 0.8 Mev Co^{58} peak in the window. The background with the conventional crystal ranged from 14 to 17 cpm and with the large diameter well, from 19 to 21 cpm. The following counts per minute per microcurie were representative. Urine beaker: 500 ml vol, 21,600; 100 ml vol, 43,000. Large well crystal: 10 ml vol, 157,000; 15 ml vol, 133,000.

Serum and urine standards were made up from each lot of material used, and the appropriate standards were counted with each set of samples. All samples from each experiment were counted on the same day without interruption or change in the spectrometer. When a series of samples required a long counting period, the standard was counted at intervals to check spectrometer stability. Almost all of the serum and urine counting was carried to an accuracy of within 10 per cent, and two-thirds of the serum samples were between 4 and 7 per cent. Some of the weaker serum samples (24 to 48 hours) could be counted to only 10 to 15 per cent accuracy without unreasonably long counting times.

Body counting. A dose of 0.17 μg (1.2 to 1.5 μc) in 1.0 ml was rapidly injected intravenously. One of two matched scintillation probes was placed over the fourth left intercostal space at the sternal border (precordium) and the other over the sixth right intercostal space at the midclavicular line angled at about 45° (liver). The collimators admitted radiation from an area 8.5 cm in diameter at 5 cm below the skin and from an area of 15 cm diameter at 15 cm depth. The ends of the collimators were in contact with the skin. Continuous recordings were made using a Baird-Atomic model 535 ratio analyzer. One probe recorded the liver counts and the other the ratio of liver/liver + precordium. Five minutes after injection the simultaneous and continuous recording was stopped, and interval body counts were continued with one probe and a scaler. The body counts using the latter setup were made over two liver sites, the sixth right intercostal space at the midclavicular line and the ninth right intercostal space at the midaxillary line; over the left kidney; and over the sacrum. The counting error with the latter setup was less than 5 per cent.

Calculations. The zero time concentration, as plotted in Figures 1, 2 and 5, was taken as microcuries injected/plasma volume. This time zero concentration was used as 100 per cent of plasma concentration in the text where plasma disappearance is discussed, in all tables, and in Figure 2. Plasma volumes were not measured but calculated to be

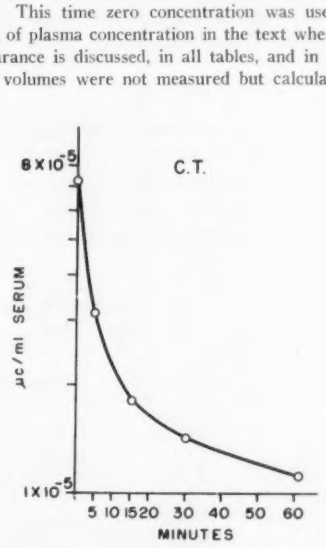

FIG. 1. PLASMA DISAPPEARANCE DURING FIRST HOUR OF A 0.043 μG INTRAVENOUS DOSE OF FREE $Co^{58}B_{12}$; CONTROL SUBJECT C.T.

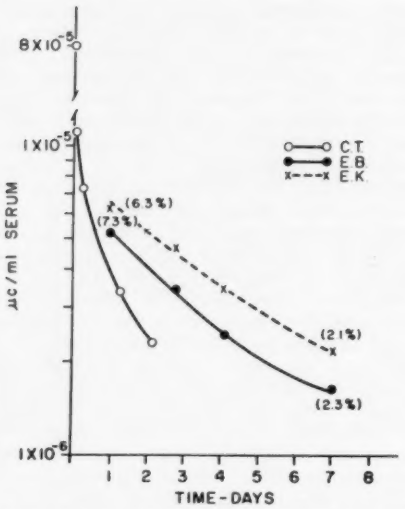

FIG. 2. PLASMA DISAPPEARANCE OF INTRAVENOUS FREE $Co^{58}B_{12}$, CONTROL SUBJECTS. Dose, C.T., 0.043 μg; dose, E.B. and E.K., 0.085 μg; % indicates the per cent of time zero concentration remaining at indicated times.

45 ml per kg body weight. As will become obvious on examination of the data, any increase in precision by actual measurement of plasma volume could have in no way altered the findings of the study. All radioactivity data were corrected for physical decay to the date that the samples were counted.

RESULTS

Disappearance of unbound intravenous dose. A dose of 0.043 μg (0.18 to 0.25 μc) of $Co^{58}B_{12}$ in 5.0 ml of saline was given rapidly (half-time, 10 to 13 seconds) to each of four subjects. The results, expressed as per cent of estimated time zero concentration remaining at intervals, are given in Table I. There was a period of very rapid loss followed by a much slower fall in radioactivity. A typical disappearance curve is given in Figure 1 and Figure 2 (C.T.). The curves of all four subjects were of similar configuration. In order to explore the period after 48 hours, two subjects were given doses of 0.085 μg, and samples were taken during the 1 to 7 day period. The results given in Figure 2 show that the slow rate of loss continued for several days.

TABLE I

*Vitamin B_{12} plasma disappearance, control subjects**

Subject	Per cent time zero concentration remaining at							
	5 min	15 min	30 min	1 hrs	6 hrs	12 hrs	24 hrs	48 hrs
E.S.	41	22	20	16	10	9.0	8.8	
L.S.	46	30	25	19	12	9.3	7.7	
H.G.	38	24	19	13	7.8	5.6	3.5	
C.T.	44	25	20	15	8.7	7.3	5.4	3.2
Mean	42	25	21	16	9.6	7.8	6.4	

* Dose in all cases was 0.043 μg $Co^{58}B_{12}$ in 5.0 ml of saline.

Disappearance of bound intravenous dose. Although in the first set of experiments the dose could have been bound by a small fraction of the total plasma volume, there was a possibility that some of the vitamin left the circulation in a free state. Doses of 7.0×10^{-3} to 1.2×10^{-2} μg (5.2×10^{-2} to 9.2×10^{-2} μc) were then mixed with various amounts and types of serum or plasma before injection. A complete set of samples was not taken in each case, but there were sufficient samples to determine the type of disappearance curve. The dose, when it was given in up to 35 ml of se-

TABLE II

Vitamin B_{12} plasma disappearance, control subjects

Subject	Dose	Dose added to:	Mixt. incub.		Binding of mixt.	Remaining in plasma at	
						15 min	1 hr
	μg		ml	min	%	%	
H.W.	0.012	Own serum	12	15		29	20
P.P.	0.007	Own serum	24	15	86	33	17
G.K.	0.012	Donor plasma	35	15	86	27	21
G.S.	0.012	Donor plasma	35	15	94	21	13
J.M.	0.012	Donor plasma	35	15	94	20	12
S.DiV.	0.010	Donor plasma	166	15	97	26	
O.S.	0.009	Donor plasma	190	15	96	24	
C.DuB.	0.007	Saline	16.5	120	80	37	20
		Own plasma	16.5				
J.Ma.	0.008	None				23	17

rum, was injected rapidly (half-time 16 to 21 seconds). When the dose was given in 166 to 190 ml of plasma, the half-times of injections were 3 minutes and 2 minutes, 30 seconds. The conditions of each experiment and the results are given in Table II. Although up to 97 per cent of the vitamin was bound prior to injection, the plasma removal was as rapid as that of the vitamin injected in a free state.

Urinary excretion. The 24-hour urinary excretion was measured in nine subjects. In some cases urine samples were taken at intervals throughout the 24 hours. The 24-hour excretion ranged from 0.1 to 1.9 per cent (mean 0.7) and almost all of the loss occurred in the first 1 to 2 hours. The dose was given in saline in four subjects, and bound to serum in five. There was no correlation between manner of administration and amount excreted.

Other body fluids. It was considered that the initial rapid loss from the serum may have been due to a rapid mixing with all extracellular fluid. Two subjects were then given 0.017 μg doses 1 hour and 3 hours, respectively, prior to a pneumoencephalogram. No radioactive vitamin was found in the cerebrospinal fluid removed, although simultaneous serum measurements showed the expected concentration. An identical dose was given to each of two subjects with cirrhosis and ascites, 2.5 and 7.5 hours prior to paracentesis. No radioactivity was found in the ascitic fluid.

Surface counting of organs. Four subjects were given an intravenous dose of free $Co^{58}B_{12}$ followed by simultaneous and continuous counting over the precordium and liver. Although absolute counting rates varied among the subjects, the distribution of the radioactivity, the time relationships, and the general proportions of activity between counting sites were the same. A typical organ uptake pattern is given in Figure 3. The hepatic radioactivity rose rapidly during a period when the precordial level was falling and reached a plateau between 1 and 2 minutes after the appearance of hepatic activity. At 5 minutes the simultaneous and continuous recording was discontinued, and counting of other organ sites at intervals was started. In Figure 3 there is an interval of 10 minutes between the plots of continuous and interval counting. From other cases it was known that little or no increase in hepatic activity would be expected during this interval. The hepatic activity of Subject A.D. rose slowly from the 15 minute level to a peak 5 days later. The activity at this peak was between 60 and 70 per cent greater than at the 2 minute level. Thus the liver took up more than half of its ultimate radioactivity in less than 2 minutes, and the remainder slowly over several days. There was also an uptake of the

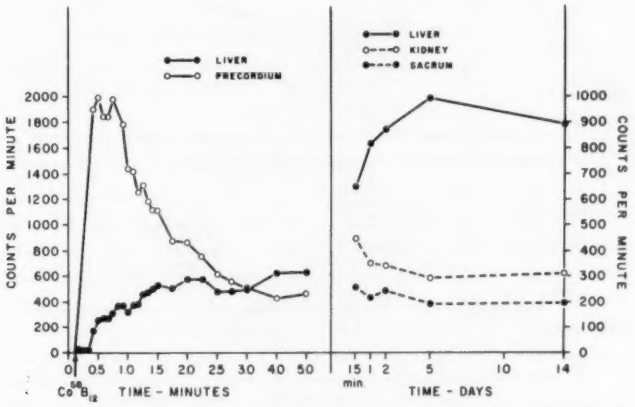

FIG. 3. BODY COUNTING, SUBJECT A.D., 0.17 μg I.V. DOSE. Section on left from continuous, simultaneous recording between 0 and 5 minutes. Section on right interval counting over three organs, 15 minutes to 14 days. Note change in vertical axis. Because of change in equipment, absolute counts are not strictly comparable between the two sections.

TABLE III

Influence of large amounts of nonradioactive ("cold") B_{12} on plasma removal of 0.043 μg Co^{58} B_{12}

Subject	"Cold" B_{12}	Route	Injection time* "cold" B_{12}	$Co^{58}B_{12}$ remaining at: Minutes 5	15	30	60	Hours 6	24	24-Hour urinary excr. of $Co^{58}B_{12}$
	μg			% dose						% dose
S.N.	1,000	I.M.	2.5 min	40	31	23	18	7.2	2.8	40
A.R.	60	I.V. Infusion	1–33 min	45	34	31	20	11	7.7	
D.R.	60	I.V. Stat dose	−15 sec	34	21	14	11	3.9	1.3	
W.H.	89	I.V.	Simultaneous†	34	19	16	12	4.3	2.1	51

* Injection time is time "cold" B_{12} given in relation to time $Co^{58}B_{12}$ given.
† $Co^{58}B_{12}$ and "cold" B_{12} were well mixed prior to injection.

kidney and sacral areas followed by a decline in activity between Days 0 and 5, a period of rising hepatic activity. The dose of 0.17 μg, which was larger than that used in the studies of plasma disappearance, was necessary to give adequate counts for continuous recording. Interval organ counts were made on three patients receiving smaller doses of 0.017 to 0.043 μg, and the same prompt hepatic uptake of half or more of the ultimate level was observed.

The plateau of hepatic uptake at 1 to 2 minutes could be considered as evidence of saturation of hepatic receptors. This possibility was investigated by giving one subject the same dose of $Co^{58}B_{12}$ (0.17 μg) mixed with 80 μg of nonradioactive B_{12}. The 5 minute and ultimate levels of hepatic activity were on the order of one-half that of the subjects receiving only $Co^{58}B_{12}$, a difference which could be accounted for by the urinary loss known to result from a dose of 80 μg (Subject W.H., Table III). There was no evidence of a hepatic saturation phenomenon.

Effect of large doses of nonradioactive B_{12}. The effect of a high plasma concentration of vitamin B_{12} on the plasma disappearance curve was studied by the following series of experiments. The in-

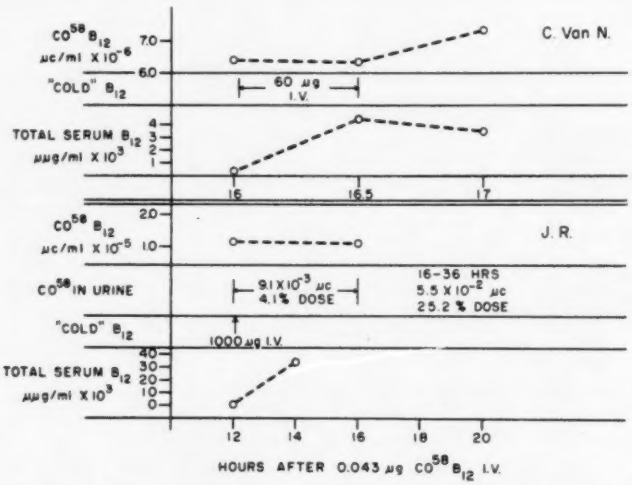

FIG. 4. THE EFFECT OF LARGE AMOUNTS OF NONRADIOACTIVE VITAMIN B_{12} ("COLD" B_{12}) ON THE $Co^{58}B_{12}$ REMAINING IN THE PLASMA 12 AND 16 HOURS AFTER I.V. ADMINISTRATION; CONTROL SUBJECTS, C. VAN N. AND J.R.

travenous dose of $Co^{58}B_{12}$ was 0.043 μg in 5.0 ml of saline in all instances.

1. In order to test the stability of the plasma B_{12} at the region of the curve where fall-off was slow, an intravenous infusion of 60 μg of nonradioactive ("cold") B_{12} was given 16 hours after $Co^{58}B_{12}$. The results given in Figure 4 (Subject C. Van N.) indicate that in spite of a rise in plasma B_{12} level to 10 times the pre-experiment level, there was no fall in the $Co^{58}B_{12}$ concentration. The rise after discontinuing the infusion was of borderline significance. In a second similar experiment (Subject J.R.), 1,000 μg was given intravenously at 12 hours (Figure 4). There was no fall in the $Co^{58}B_{12}$ plasma level over a 4-hour period although there was a rise in the total B_{12} plasma level of 70 times that of pre-experiment. Four per cent of the dose of $Co^{58}B_{12}$ was excreted in 4 hours. Had all of the excreted $Co^{58}B_{12}$ come from the plasma, there would have been a fall in $Co^{58}B_{12}$ plasma level to 65 per cent of the starting level, which did not happen. The vitamin in the urine either came from the plasma and was exactly replaced with tissue vitamin B_{12}, or it moved from tissue to urine without measurable change in plasma level.

2. Three subjects received nonradioactive vitamin B_{12} at various times in close relation to the $Co^{58}B_{12}$ (Table III). When given after the $Co^{58}B_{12}$, it had no measurable influence on the $Co^{58}B_{12}$ curve except possibly after 6 hours (Subject S.N.). Nonradioactive B_{12} given before the $Co^{58}B_{12}$ (Subject D.R.) resulted in speeding up of removal of the $Co^{58}B_{12}$, but the change was slight.

3. Subject W.H. was given both $Co^{58}B_{12}$ and nonradioactive B_{12} simultaneously in the form of 7.0 ml of a previously prepared mixture in saline. He received a total of 89 μg of vitamin B_{12} (injection half-time 6 seconds). There was some deviation from the type of curve obtained with small doses, but the changes were surprisingly minor (Table III) and could have been produced solely by the 51 per cent loss in the urine.

DISCUSSION

The present study is primarily concerned with the description of plasma vitamin B_{12} disappearance in the subject without disturbance of vitamin B_{12} metabolism. Mathematically, the vitamin B_{12} disappearance curves obtained above could be analyzed in more than one way, but the concept of a multi-compartment closed system (7) seemed to best fit the data. The equation for such a system of four compartments is:

$$C = ae^{-k_1 t} + be^{-k_2 t} + ce^{-k_3 t} + de^{-k_4 t} + f,$$

where C = the plasma concentration; a, b, c, d, and f are constants; and k_1, k_2, k_3, and k_4 are the summation of constants expressing the fractional change per unit time for separate processes. All of the curves could be broken down into four such components. For example, the curve of Subject C.T. was analyzed by the procedure of Veall and Vetter (7) to give the following values of k as the fractional part removed per hour: $k_1 = 7.4$; $k_2 = 0.80$; $k_3 = 0.10$; $k_4 = 0.017$. The biological significance of this analysis is a matter of conjecture, but it is useful in discussing the curves. The rapid component of the disappearance curve was similar following doses ranging from 7×10^{-3} to 89 μg, although the data do not permit the conclusion that rates or physiological mechanisms were identical. With the doses of 0.043 μg and less, all of the $Co^{58}B_{12}$ in the serum was in the bound form. In the study using an 89 μg dose, almost all was in the free form, but similar curves were obtained in both studies. In the pre-binding studies, 80 to 97 per cent pre-injection binding was achieved, but the vitamin was still no better retained in the circulation. These findings do not support the concept that the greater the binding capacity of the plasma, the slower the loss from the circulation.

The distribution of the initially rapidly disappearing tracer dose could be only partially determined. It was not excreted and was not detected in cerebrospinal fluid or ascitic fluid. There was evidence of rapid accumulation in the liver and kidney. Liver accumulation then continued at a slower rate after the plasma $Co^{58}B_{12}$ had fallen to a low level. Possibly, that vitamin rapidly taken up by tissue was intracellular but not in its ultimate intracellular form. Evidence of such a concept is found in the recent work of Strength, Alexander and Wack (8). They observed a fall in rat kidney homogenate B_{12} and supernatant B_{12} but a rise in the mitochondrial content over the period of 0.5 day to 12 days to 30 days after the last

of five doses of 1.0 μg. The rat liver homogenate B_{12} rose over the same period, and while the B_{12} content of both the supernatant and mitochondrial fractions increased, the latter showed the greater increase.

The initial phase of disappearance was virtually complete in an hour or less. The events following immediately were less well defined. A substance which moves among several compartments, as in the type of system being used as a model in this discussion, both enters and leaves a compartment. Re-entry into a compartment such as the plasma could not be detected if the total rate of loss was continually greater, and no evidence of re-entry into the plasma was detected in the control subjects. The two experiments made 12 and 16 hours after the injection of $Co^{58}B_{12}$ indicate that by this time the vitamin was in a state differing markedly from that shortly after administration. The level of $Co^{58}B_{12}$ did not fall as large amounts of non-radioactive B_{12} passed through the plasma, but $Co^{58}B_{12}$ was still available from some source for urinary excretion and hepatic uptake.

The fourth component of the equation which predominated after 24 hours may be significant in relation to total body turnover. This component is evident by inspection of all curves carried to 24 to 48 hours but is best seen in the curves of the two patients followed beyond 48 hours (Figure 2). The k_4 for Subject E.B. was 8.0×10^{-3} per hour with a half-time of 82 hours. Assuming that the daily plasma turnover of vitamin B_{12} equals the fraction removed per day times total plasma B_{12} (micromicrograms per milliliter) times plasma volume (milliliters), Subject E.B. turned over 0.18 μg per day between Days 1 to 7. There are too many unknown factors to permit the conclusion that this figure represents the plasma turnover of normally metabolized B_{12}, but the order of magnitude is in keeping with the general concept that body B_{12} turns over slowly.

A significant point is brought out by comparing the 48 hour $Co^{58}B_{12}$ level of a subject receiving 0.043 μg (C.T.) with that of the subject receiving 89 μg (W.H.). C.T. bound 3.2 per cent of the dose in his plasma, or less than 1.0 $\mu\mu$g per ml. W.H., who lost 50 per cent of the dose in the urine, bound either 0.50 per cent or 2.4 per cent, depending upon whether the serum level was measured by radioactivity or bioassay, which was on the order of 1,000 times that bound by C.T. Obviously the amount of B_{12} bound in the plasma at 48 hours (body distribution was relatively fixed by this time) was determined by a process more complex than a simple addition of vitamin B_{12} to a plasma with unsaturated binding sites.

The present work should also be discussed in terms of the more restricted concept of binding presented by Miller and Sullivan (4). Subject C.DuB. (Table II) received 7.0×10^{-3} μg of B_{12} previously mixed with 16.5 ml of his own plasma and incubated for 2 hours. Using 650 $\mu\mu$g per ml as the binding capacity of the native binding protein (BP) (4), this subject could bind 460 $\mu\mu$g per ml in addition to that already present (650 $\mu\mu$g per ml minus the bound native B_{12}, 190 $\mu\mu$g per ml). Since 420 $\mu\mu$g was added per ml, all could have been bound to the native BP. However, actual determination of the binding site is necessary in such experiments, for possibly other binding sites preferentially bind the B_{12} *in vitro*. Handling of plasma prior to *in vitro* binding may alter the BP, although this did not appear to be the case with the chronic myelogenous leukemia sera studied by Miller and Sullivan. Applying the same type of calculation, Subject J.Ma. (Table II), who received the dose in saline, would be able to bind all of the dose in 29 ml of plasma. Even Subject C.T. (Table I), who received a larger dose in saline, would be able to bind the dose completely with 112 ml of plasma. It would then appear that these two subjects could have promptly bound the added vitamin B_{12} to the BP *in vivo*. One cannot assume that such a binding does take place. Actual measurements of binding site must be made and compared with the binding resulting from intramuscular and oral administration. It is not known whether intravenous administration simulates natural intake. Orally induced vitamin may bind to BP immediately on entry into blood or it may bind to another site and shift to BP either within the circulation or within a specific tissue.

If adequate techniques can be developed, further studies of the chemical state of the B_{12} in plasma would also answer the question of whether the Co^{58} in the plasma various times after a dose remains in the form of $Co^{58}B_{12}$. Such a question is crucial in the interpretation of the present study. The amounts in plasma in the present study were

too small to permit analysis. However, several investigators studying dynamics of larger doses have used various direct or indirect means to determine whether labeled B_{12} remains as such after administration. No one has presented any evidence of the *in vivo* separation of the radioactive cobalt from the B_{12}.

SUMMARY

The plasma removal of intravenous doses of 7.0×10^{-3} to 4.3×10^{-2} μg of cobalt^{58} vitamin B_{12} was studied in control subjects. Initially the vitamin was removed rapidly, less than 20 per cent remaining in 1 hour. This loss did not represent excretion but was due to entry into tissue. Complete *in vitro* serum or plasma binding of the vitamin prior to administration did not influence the rate of disappearance. An increase in dose of 2,000-fold did not significantly change the fraction removed per unit time during the initial phase of loss. Twelve to 16 hours after administration the vitamin in the blood was more firmly fixed than some of the tissue B_{12}. After 24 hours a slow decline in plasma activity continued for several days.

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THE EFFECT OF SOME MERCAPTANES UPON A MACRO-CRYOGELGLOBULIN; MODIFICATIONS INDUCED BY CYSTEAMINE, PENICILLAMINE AND PENICILLIN *

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Although macroglobulinemia of Waldenström is a rare disorder, the physicochemical modification of macroglobulins therein has broad implications, since similar large proteins may be involved in the pathogenesis of symptoms associated with a variety of relatively common diseases; i.e., Rh isosensitization, hemolysis due to cold agglutinins, and so on. Also, a number of normally occurring proteins (properdin, isohemagglutinins, and others) are macroglobulins.

In macroglobulinemia of Waldenström the pathological proteins are responsible for the increased serum viscosity which is usually present (1). In certain instances the macroglobulins also possess the properties of cryoglobulins or cryogelglobulins, i.e., they precipitate or gel at temperatures lower than 37° C (2). These abnormal proteins (paraproteins) may be responsible for the production of Raynaud's phenomenon, ulcerations or gangrene. High environmental temperature may be somewhat helpful in preventing the damaging effects of cryo or cryogelglobulins. Plasmapheresis and exchange transfusions have not been found to be satisfactory in the elimination of the paraproteins over long periods of time (3, 4). Therefore, another avenue of investigation into phenomena related to these substances has been explored.

Deutsch and Morton (5) and Glenchur, Zinneman and Briggs (6) have demonstrated that mercaptanes will depolymerize macroglobulins. The addition of 0.1 M cysteine at pH 7.4 to a macroglobulin with 18S, 25S and 32S components produced conversion to a single 6S component. Following removal of cysteine by dialysis, there was reaggregation of the proteins with the formation of two different types, one of 13S and the other of 18S (5). Heimer and Federico (7) have conclusively demonstrated that the macroglobulins representing the rheumatoid arthritis factor (19 and 22S components) can be depolymerized to a 7S unit with cysteine. This reduction was associated with loss of its hemagglutination properties. Despite subsequent reaggregation, hemagglutination could not be demonstrated. Isliker (8) treated the physiological 19S α-2-macroglobulin with cysteamine; thereafter, the ultracentrifugal pattern revealed a 6S component. Depolymerization was also indicated by an immediate sharp drop (more than 50 per cent) of the original relative viscosity. In contrast to the effect upon macroglobulins, similar treatment of normal 7S γ-globulins did not produce any change of the ultracentrifugal or viscosity patterns. It has been shown that cysteamine can convert properdin from a 23 to 25S component to a 6S fraction (8).

Methods which might induce depolymerization or abolish cryogel properties of a paraprotein associated with macroglobulinemia of Waldenström were investigated.

MATERIALS AND METHODS

The experimental material for this study was provided by a patient with macroglobulinemia of Waldenström proven by bone marrow examination, serum viscosity, and immunological and ultracentrifugation studies. The serum exhibited the properties of a cryogelglobulin between 13° and 20° C (2). The S_{20} value of this protein was 19S and the macroglobulin fraction comprised 38 per cent of the total serum proteins; total γ-globulin content of the serum was 7.6 g per 100 ml. Electrophoretically, the macroglobulin was a homogeneous γ-globulin of the intermediate type. The intrinsic viscosity [specific viscosity [1] (η rel. -1) divided by the concentration of serum protein in grams per 100 ml, at infinite dilution] of this macrocryogelglobulin was ap-

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[1] Relative viscosity, η rel.

proximately 0.125. The axial ratio of the 95 per cent purified macroglobulin was approximately 11.5 (dehydrated) and 8.5 (hydrated). The Sia (euglobulin) test was strongly positive [3 + (2)].

Freshly drawn heparinized whole blood and fresh serum as well as aged serum (stored at −20° C for 7 days) were used in these studies. In addition to the whole blood and serum from the patient with macroglobulinemia, heparinized whole blood samples from 7 patients with multiple myeloma and from 4 normal persons were examined.

Chemical substances used were DL-penicillamine (DL-dimethylcysteine),[2] cysteamine (β-mercaptoethylamine),[3] penicillin G potassium (Squibb) and Benemid (Merck).

Antiserum against the patient's serum that was rich in macrocryogelglobulin (aB) was prepared by injecting 1 ml of fresh macrocryogelglobulin serum into the marginal ear veins of a 2 kg rabbit every other day during a 10 day period. After 10 additional days, the rabbit was exsanguinated under sterile conditions by percutaneous cardiac puncture. The serum (aB) was stored at +4° C.

1. Studies of the effects of DL-penicillamine upon serum viscosity, gel formation, Sia test, formol gel test and serum protein content

a. DL-Penicillamine (10 mg in 0.2 ml of 0.9 per cent sodium chloride solution) was added to 2 ml of aged macroglobulin serum. This was incubated at 37° C for 15 minutes. Determination of the serum viscosity was performed at 20° C using an Ostwald viscosimeter of 2 ml volume. The relative viscosity was calculated by dividing the flow time of serum in seconds by the flow time of distilled water in seconds. Paper electrophoretic examination of the serum samples was carried out (Spinco Durrum, model R, Series C). Measurements of η rel. and electrophoretic examinations as well as determination of the total serum proteins were made immediately before and after and 24 hours (kept at +4° C) following incubation.

b. DL-Penicillamine was administered orally (250 mg every 6 hours in capsules) for 19 days. Serial determinations of serum viscosity (20° C) were carried out and the concentrations of the total serum proteins and globulin fractions were determined.

The serum samples were also tested for gel formation (at 4°, 13° and 20° C). The Sia test and formol gel test were performed. The Sia test was performed and graded as follows (2). One drop (0.1 ml) of serum was added to 10 ml of electrolyte-free water using a Pasteur pipet (rinsed in electrolyte-free water). The formation of slight turbidity within 10 seconds was graded 1+. Moderate turbidity and heavy (milky) turbidity were graded 2+ and 3+, respectively. Readings were performed within 10 seconds. The technique used for the formol gel test was as follows. To 0.5 ml of serum 1 drop of 40 per cent formalin was added using a Pasteur pipet. Gel formation within 2 minutes was considered to be a positive formol gel test.

2. Studies of the effect of penicillin on viscosity, gel formation, Sia test, formol gel test, thermosedimentation rate and protein content of serum

a. To 2 ml of freshly drawn serum from the patient with macroglobulinemia, 0.2 ml of 0.9 per cent sodium chloride solution was added. This sample served as a control. To a second sample of macroglobulin serum, 20,000 U of penicillin G potassium (Squibb) dissolved in 0.2 ml of 0.9 per cent sodium chloride solution, was added. The serum samples were incubated at 37° C for 30 minutes. This procedure was also applied to aged serum. Immediately before and after incubation with penicillin G, the sera were tested for gel formation at 4°, 13° and 20° C; the viscosity was determined at 20° C; the Sia test and formol gel test were performed.

b. Ten million U of penicillin G potassium in 250 ml of 5 per cent glucose in water was infused intravenously within 1 hour. Viscosity of the serum was determined immediately before and after the injection and at 30 and 90 minutes thereafter. The sedimentation rates were determined at 37° C immediately before and after penicillin administration. Determination of total serum proteins and paper electrophoretic examination were carried out on serum samples removed immediately before and after penicillin infusion.

c. The same procedure was repeated with penicillin G potassium dissolved in only 100 ml of 5 per cent glucose in water.

d. The procedures were identical with those under 2*b* except for the additional administration of Benemid in a dose of 250 mg every 6 hours, begun 12 hours prior to the infusion of penicillin and continued to the end of the tenth day. The serum viscosity was measured immediately before and after the administration of penicillin and at time intervals of 1, 2, 24, 48 and 72 hours. The thermosedimentation rates were obtained by determining the sedimentation rates of heparinized blood samples simultaneously at 4, 13, 25 and 37° C (Wintrobe).

3. Studies of the effect of cysteamine upon the viscosity, gel formation and Sia test

a. Two-tenths ml of freshly prepared cysteamine solutions of varying concentrations (1.0, 0.1 and 0.01 per cent) was added to 2 ml of aged serum. To a fourth serum sample which served as a control, 0.2 ml of 0.9 per cent sodium chloride solution was added. All samples were incubated at 20° C for 15 minutes; the η rel., gel formation and Sia test were determined immediately before and after incubation and 24 hours later (serum was stored at 4° C).

4. Studies of the effect of DL-penicillamine, penicillin G potassium and cysteamine upon the immunophoretic properties of the macrocryogelglobulin

The Ouchterlony agar diffusion technique (9) was employed to study the influence of the DL-penicillamine, peni-

[2] Aldrich Chemical Co., Milwaukee, Wis.

[3] K. & K. Laboratories, Jamaica, N. Y.

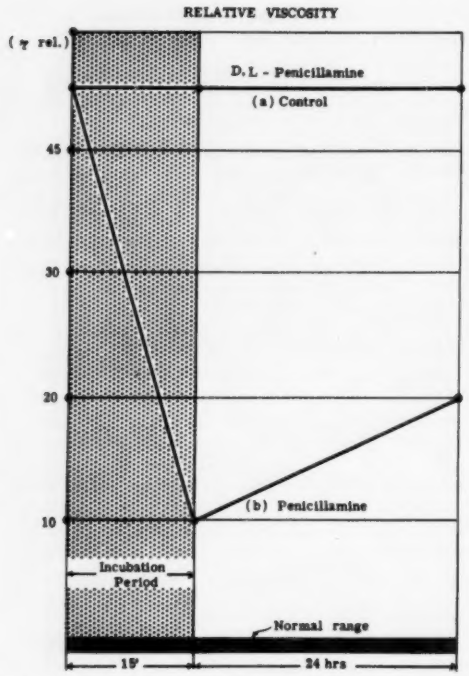

FIG. 1. INFLUENCE OF *IN VITRO* ADDITION OF DL-PENICILLAMINE (10 MG DISSOLVED IN 0.2 ML OF 0.9 PER CENT NaCl SOLUTION) UPON RELATIVE VISCOSITY OF AGED MACROCRYOGEL SERUM (2 ML; SEE TEXT).

cillin G potassium and cysteamine upon the macrocryogelglobulin. In Petri dishes containing 2 mm thick layers of 1.5 per cent purified agar gel (Difco-Nobel agar) the precipitin reactions were observed between a rabbit antimacrocryogelglobulin serum (aB), the macrocryogelglobulin serum (B), and an anti-γ-globulin serum (aG).

a. The patient's serum served as a control.

b. In a second series, DL-penicillamine was added to the agar at 40° C (the final concentration of penicillamine was 1 per cent) and rabbit antimacrocryogelglobulin serum (0.6 ml per well) was allowed to react with the macrocryogelglobulin serum (0.4 ml per well). Readings were made after 24 hours.

c. In a third series penicillin G potassium was incorporated into the agar gel. The final concentration of penicillin was 1 per cent.

d. In a fourth group, cysteamine was added to the agar gel (final concentration of cysteamine was 1 per cent) and rabbit antihuman macrocryogel serum was allowed to react with macrocryogelglobulin. In order to prevent dissociation of the mercaptane cysteamine, the Petri dishes were kept in an atmosphere of pure nitrogen.

To assure comparable results, each group of *in vivo* studies was performed only after viscosity, sedimentation rate, Sia test and gel formation had returned to pretreatment levels.

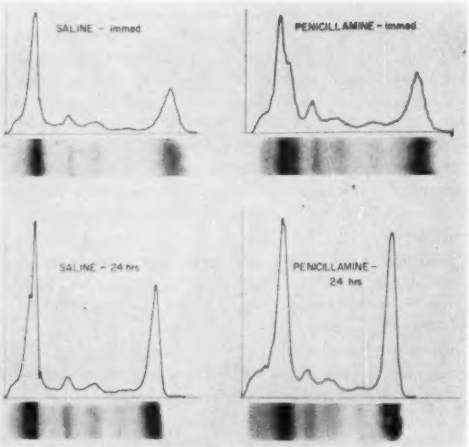

FIG. 2. ELECTROSEROGRAM; CONTROL SERUM AND PENICILLAMINE-TREATED SERUM IMMEDIATELY BEFORE AND AFTER INCUBATION AS WELL AS 24 HOURS LATER (SEE TEXT).

RESULTS

1. *DL-Penicillamine*

a. The addition of DL-penicillamine to macroglobulin serum resulted in a decrease of the η rel. from 47.4 to 10.2 immediately following incuba-

TABLE I

Effect of addition of DL-penicillamine to macroglobulin serum upon total serum proteins and electrophoretic partitions.

	T.S.P.*	Albumin	Globulins			
			α_1	α_2	β	γ
	g %	%	%			
1. Control samples						
Immed. before incubation	8.7	28.5	6.5	10.1	10.8	44.1
Immed. after incubation	9.5	29.9	5.2	8.6	9.6	47.7
24 Hrs after incubation	9.2	28.6	5.5	8.6	9.0	48.3
2. Test samples						
Immed. before incubation	9.5	29.4	8.1	10.1	9.7	42.7
Immed. after incubation	9.6	33.6	4.1	7.9	6.7	47.7
24 Hrs after incubation	9.2	30.3	5.6	8.2	8.4	47.5

* Total serum proteins.

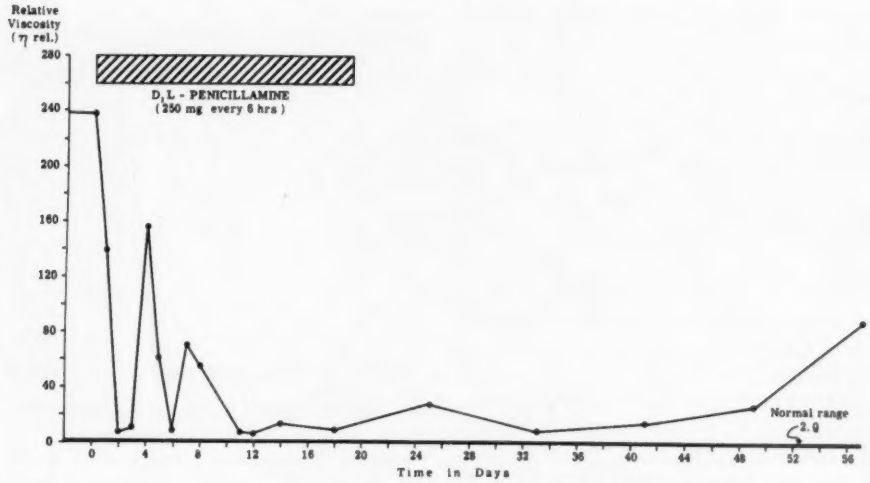

FIG. 3. INFLUENCE OF ORALLY ADMINISTERED DL-PENICILLAMINE (250 MG 4 TIMES DAILY) UPON RELATIVE VISCOSITY OF SERUM (SEE TEXT).

tion and 20.7 after 24 hours (Figure 1). Penicillamine-treated serum revealed splitting of the homogeneous main γ-globulin fraction when electroserogram was obtained immediately after incubation. Twenty-four hours later (upon standing at 25° C) again a homogeneous γ-globulin fraction was seen. The control sample (saline) did not reveal these changes (Figure 2). The absolute values for the total serum proteins and the relative values for the albumin and globulin fractions are shown in Table I.

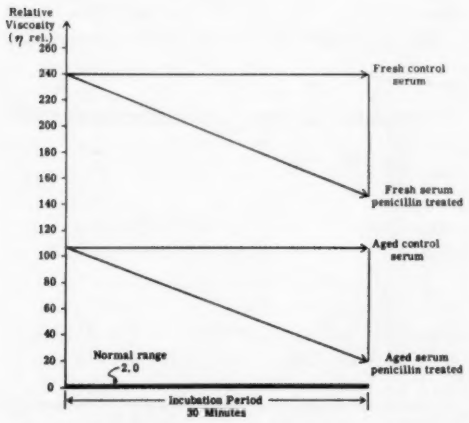

FIG. 4. INFLUENCE OF THE *IN VITRO* ADDITION OF PENICILLIN G POTASSIUM UPON RELATIVE VISCOSITY OF FRESH AND AGED SERUM. Two-tenths ml of 0.9 per cent NaCl solution containing 20,000 U of penicillin G potassium was added to 2 ml of serum; sera were incubated at 37° C for 30 minutes.

b. Oral administration of DL-penicillamine provoked a successive decrease of the η rel. from 239 to 6.9 (97 per cent) within two days (Figure 3). Except for transient increases of η rel. on Days 4 and 7, the viscosity remained at levels close to the normal range (2). Despite the discontinuance of penicillamine on Day 19, no substantial increase of viscosity appeared within the following 30 days. The absolute values of total serum proteins and relative values of the γ-globulin fractions (in parentheses) were: 9.4 g per 100 ml (52.2 per cent) one day prior to the beginning of penicillamine treatment; during the treatment and post-treatment periods the following values were obtained: first day, 9.5 g per 100 ml (46.5 per cent); sixth day, 9.6 (43.4); thirty-fifth day, 8.8 (37.9); fifty-third day 9.1 (40.7) and seventy-fifth day, 9.2 (44.4). The main γ-globulin fraction (homogeneous macroglobulin fraction) was "split" on the sixth day of the treatment period. Gel formation of the serum was abolished and the Sia test became negative on the second day of treatment. There was no change in the formol gel test throughout the test period. No major increase of

the η rel. was noted through the fifty-seventh day of observation.

2. Penicillin G

a. Following *in vitro* incubation of the serum with 20,000 U of penicillin G potassium, a significant decrease of relative viscosity was observed. In treated fresh serum, the η rel. decreased from 240 to 148 (38 per cent); in treated aged serum, the relative viscosity decreased from 107 to 20.2 (81 per cent, Figure 4). Gel formation occurred at temperatures between 13° and 20° C before incubation in both fresh and aged sera, but following *in vitro* incubation with penicillin it did not appear at 4°, 13° or 20° C. The Sia test was strongly positive (3+) in both serum samples before incubation, but was only slightly positive (1+) following incubation with penicillin. The formol gel test remained positive.

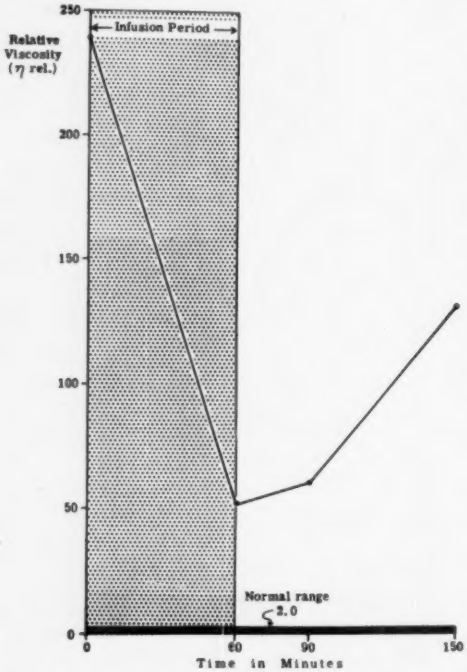

FIG. 5. INFLUENCE OF THE INTRAVENOUS ADMINISTRATION OF PENICILLIN G POTASSIUM UPON RELATIVE VISCOSITY OF SERUM. Ten million U of penicillin G diluted in 250 ml of 5 per cent glucose in water was given intravenously within 60 minutes.

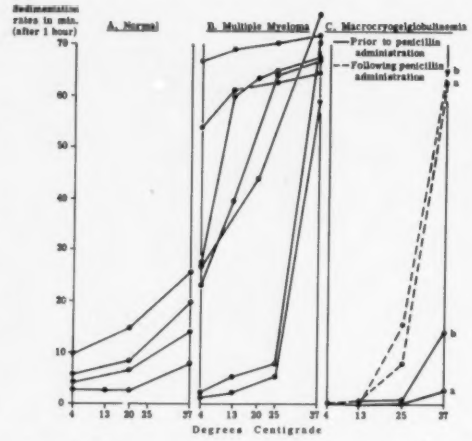

FIG. 6. THERMOSEDIMENTATION RATES. At 4°, 13°, 20°, 25°, and 37° C in: A, normal individuals [4]; B, multiple myeloma [7]; C, macrocryogelglobulinemia (—— prior to penicillin administration, - - - - following penicillin administration).

b. The intravenous infusion of 10 million U of penicillin G potassium was followed by an immediate sharp drop of the relative viscosity of the serum from 243 to 54.8 (77 per cent, Figure 5). Ninety minutes following the completion of the infusion there was an increase of viscosity to 130.

The sedimentation rate prior to the penicillin administration was quite low (3 mm after 1 hour at 37° C); whereas after penicillin infusion the

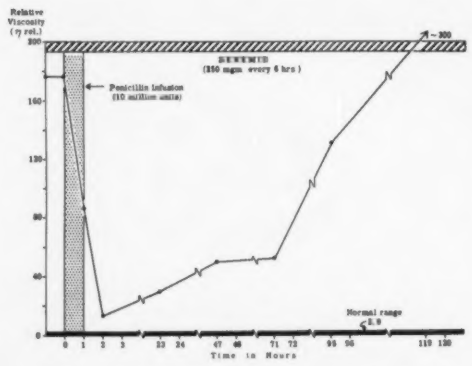

FIG. 7. RELATIVE VISCOSITY OF SERUM BEFORE AND AFTER THE INTRAVENOUS ADMINISTRATION OF 10 MILLION U OF PENICILLIN G POTASSIUM. In 250 ml of 5 per cent glucose in water combined with Benemid (250 mg every 6 hours, orally).

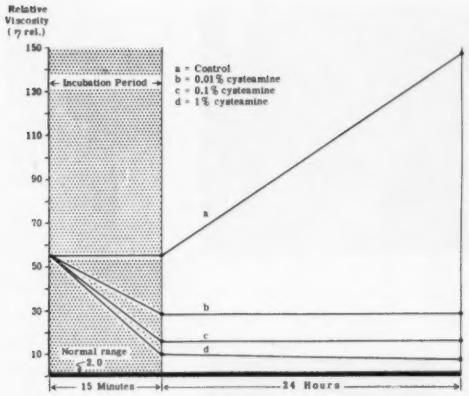

FIG. 8. INFLUENCE OF VARYING CONCENTRATIONS OF CYSTEAMINE UPON RELATIVE VISCOSITY OF AGED SERUM *IN VITRO* IMMEDIATELY AND 24 HOURS AFTER INCUBATION (SEE TEXT).

sedimentation rate increased to 64 mm after 1 hour at 37° C (Figure 6C, a).

c. The infusion of 10 million U of penicillin G dissolved in 100 ml of 5 per cent glucose in water produced a decrease of serum viscosity almost identical with that seen following the infusion of penicillin in 250 ml of 5 per cent glucose in water (*2b*). The sedimentation rate (37° C) became rapid after penicillin infusion (from 17 to 67 mm after 1 hour, Figure 6C, b).

d. A third infusion of 10 million U of penicillin G potassium was combined with the administration of Benemid. Again, the drop in relative viscosity was striking (from 175.5 to 85.9), but no rebound phenomenon occurred. Indeed, the viscosity decreased further to 14.0 during the next hour with a total reduction of 92 per cent in relative viscosity at the end of 2 hours. Follow-up studies revealed a slight increase of viscosity during the next three days; after five days the original viscosity had been exceeded (Figure 7).

The pattern of the thermosedimentation rate resembled that seen in the preceding experiment (0 mm at 4°, 0 mm at 13°, 0 mm at 25°, and 2 mm at 37° C before penicillin and 0 mm at 4°, 1 mm at 13°, 3 mm at 25°, and 62 mm at 37° C, 1 hour after penicillin administration).

3. Cysteamine

a. The relative viscosity of the aged serum before incubation with cysteamine was 56. Immediately following incubation the following values were obtained: control (a) 56; sample (b) (0.01 per cent cysteamine) 29; sample (c) (0.1 per cent cysteamine) 16; sample (d) (1.0 per cent cysteamine) 11. After 24 hours' incubation the values were as follows: control (a) 146; sample (b) 28.2; sample (c) 16; sample (d) 8.5 (Figure 8). Gel formation was abolished between 4° and 20° C and the Sia test became negative in all three treated serum samples.

b. Essentially the same pattern was obtained in fresh serum (Figure 9).

4. The effect of DL-penicillamine, penicillin G potassium and cysteamine upon immunophoretic properties

Ouchterlony plates

a. The control plates revealed a γ-globulin line between the antigen (B) and the antibody wells (aG and aB) and a macrocryogelglobulin precipi-

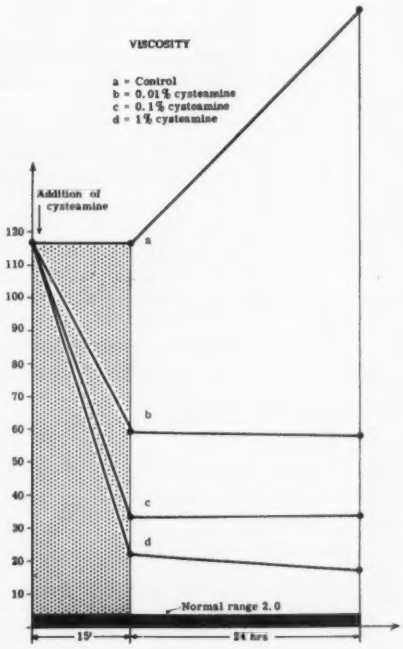

FIG. 9. INFLUENCE OF VARYING CONCENTRATIONS OF CYSTEAMINE UPON RELATIVE VISCOSITY OF FRESH SERUM (SEE TEXT).

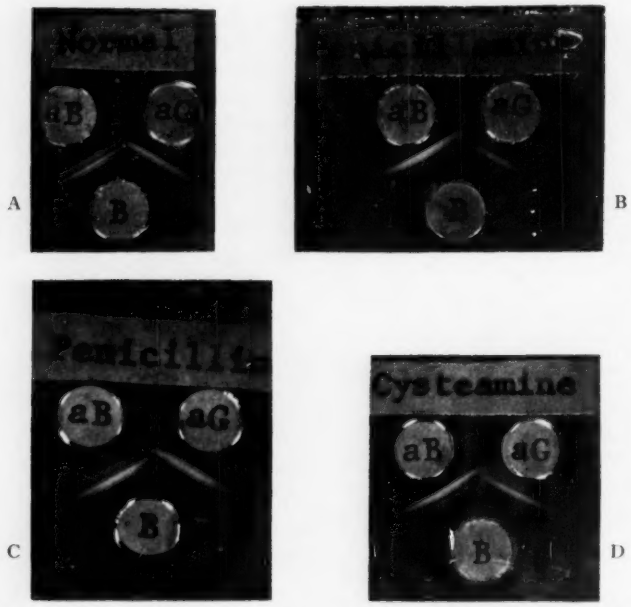

FIG. 10. OUCHTERLONY AGAR GEL DIFFUSION TESTS. The samples were allowed to diffuse from their respective wells. At the site of confluence precipitin lines were formed [aB: anti-human macrocryogelglobulin-serum; B: macrocryogelglobulin-serum; aG: anti-γ-globulin-serum (Behring)].

A. *Control.* The precipitin line close to the antibody well (aB) represents the γ-globulin line; the well-outlined line in the middle between the aB and B wells is the macroglobulin precipitin line; the precipitin line between B and aG represents the γ-globulin line.

B. Following the addition of DL-*penicillamine* to the agar gel, the precipitin lines of the macroglobulin have joined those of γ-globulin, indicating a change in diffusion rate and immunological affinity to γ-globulin.

C. The addition of *penicillin* to the agar gel reveals no changes as compared with the control plates (A).

D. *Cysteamine* has resulted in changes of mobility of the macrocryogelglobulin which are comparable with those seen after the addition of DL-penicillamine.

tin line between the antibody (aB) and antigen wells (Figure 10A).

b. The addition of DL-penicillamine resulted in the disappearance of the macroglobulin precipitin line and an increase of the γ-globulin line which shares partial antigenicity with the gamma line between B and aG (Figure 10B).

c. Penicillin G potassium did not evoke any obvious changes of the precipitin pattern (Figure 10C).

d. The addition of cysteamine to the agar gel resulted in disappearance of the macroglobulin precipitin lines, and an increase of the γ-globulin precipitin lines was noted (Figure 10D).

DISCUSSION

DL-Penicillamine is used as a chelating agent in heavy metal poisoning (10) and in Wilson's disease (11–13). Toxic side effects, such as inhibition of growth in animals (14), interference with vitamin B_6 utilization (12, 14–16) and the activity of some hepatic transaminases (16, 17) as well as dermatological manifestations (18) have been produced by the L-form of penicillamine.

D-Penicillamine appears to be nontoxic (14). The high price of this compound, however, limits its liberal clinical use. Penicillin is partly metabolized into D-penicillamine (12, 19–21). The above observations indicate that both penicillin (or a breakdown product of penicillin) and DL-penicillamine produce pronounced changes in the physical characteristics of the serum both *in vivo* and *in vitro*. Also, after penicillamine and penicillin therapy there was marked clinical improvement, which was reflected by disappearance of cold intolerance, cessation of hemorrhagic diathesis with normalization of clotting status, increase in appetite and weight, and generalized feeling of well-being.

Following depolymerization of macroglobulins with mercaptanes, there is usually a pronounced rebound of the η rel. (Figures 1, 5, 7). Thus, the η rel. may even exceed the original value within one hour (8). The pronounced rebound of η rel. following the infusion of penicillin G imitates this characteristic feature of depolymerization although the alterations in the physical characteristics of the serum reported herewith could theoretically be due to some molecular modification other than depolymerization. The prolonged decrease of serum viscosity and increase in thermosedimentation rate following the penicillin infusion with Benemid suggest increased retention and sustained action of either penicillin or a metabolite, such as penicillamine. The altered pattern of thermosedimentation rate following treatment with penicillin and penicillamine both *in vitro* and *in vivo* is similar to that seen in some untreated myeloma sera with 7S paraproteins (Figure 6B).

The failure of the relative viscosity to return completely to normal may be due either to persistence of macroglobulins or to an increased amount of 7S globulins. In the presence of macroglobulins without cryo or cryogel properties the serum viscosity is higher than normal. However, it has been conclusively shown that increased quantities of 7S γ-globulins can also produce slightly increased η rel. This is particularly true in the presence of 7S paraproteins, such as multiple myeloma protein (22). Disintegration of a pathological macroglobulin may result in the production of such a small sized paraprotein.

On theoretical grounds, removal of the macroglobulin from the blood could explain these findings. Unchanged total serum protein and electrophoretic results of the examined serum samples immediately before and after treatment with penicillamine *in vitro* and *in vivo* rule out this possibility. Furthermore, Isliker's findings (8) of pronounced reduction in the serum viscosity with subsequent rebound following the depolymerization of the α_2-macroglobulin are similar to observations made following treatment of our serum samples with penicillamine and penicillin.

The change in the Sia test readings may be attributed to reduction in the molecular size of the macroglobulin, since the precipitation of the paraproteins appears to be independent of the cryo or cryogel properties. According to Steel (23) the Sia test can be employed in testing the stability of macroglobulins. In contrast, the abolishment of gel-forming characteristics following treatment with penicillin, penicillamine and cysteamine can be explained by inactivation of the cryogel properties of the macroglobulin without additional dissociation of the macromolecule.

Hitzig, however, has been able to demonstrate (24) a slow spontaneous dissociation of this macrocryogelglobulin of an aged serum sample. The addition of cysteamine to the serum and to the purified macrocryogelglobulin greatly accelerated this dissociative process. This finding is comparable with the results of Métais and Warter [cited by Hitzig and Isliker (25)] who found a slowly decreasing high molecular fraction from the serum of a patient with macroglobulinemia merely after dilution. Steel (23) also observed spontaneous dissociation of isolated macroglobulins at room temperature and at 37° C. We (26) have encountered decreasing serological activity as reflection of depolymerization of macromolecular cold agglutinins during a storage period of four months at − 20° C. Spontaneous dissociation probably accounts for the lower η rel. of aged macroglobulin serum, as seen in samples of aged macrocryogelglobulin serum.

The profound decrease of relative viscosity following incubation of the serum with cysteamine may thus reflect dissociation of the macroglobulin (8). Reduction of viscosity, abolishment of gel formation and conversion of a strongly to a weakly positive or negative Sia test following *in vitro* treatment of the macroglobulin serum with penicillamine and penicillin, as well as following adminis-

tration of these agents to the patient, were similar quantitatively and qualitatively. Both penicillamine (26–28) *in vivo*, and cysteamine (8, 24, 25) *in vitro* are able to depolymerize macroglobulins as demonstrated by ultracentrifugation. The marked changes of viscosity, Sia test and gel formation in the macroglobulin are certainly compatible with dissociation of the macroglobulin. The electrophoretic changes of penicillamine-treated serum favor this suggestion. The resemblance of the reaction pattern of penicillin G upon the macroglobulin is suggestive of the same mechanism. The inactivity of penicillin in the agar gel diffusion test supports, however, the concept that a metabolite of penicillin (D-penicillamine?) may be responsible for the *in vivo* action. The conversion of penicillin into D-penicillamine appears to be dependent upon certain agents present in serum. The lack of activity of penicillin, incorporated into agar-gel, could thus be explained.

Regardless of the underlying mechanism, this biochemical approach to macroglobulinemias, particularly those with cryo or cryogel characteristics, may be of clinical value. This is supported by the marked clinical improvement of the patient under study. Description of this observation will be reported elsewhere.

SUMMARY

The effects of penicillin G potassium, DL-penicillamine and cysteamine upon a macrocryogelglobulin were investigated. Changes of serum viscosity, thermosedimentation rates, gel formation, Sia test, total serum protein, paper electrophoretic characteristics and immunophoretic properties served as indicators.

Administration of DL-penicillamine produced a marked sustained decrease of relative viscosity and an increase of sedimentation rate. DL-Penicillamine, added to macrocryogelglobulin serum *in vitro* resulted in marked decrease of relative viscosity, widening of macroglobulin fraction in electrophoretic pattern, and a negative Sia test and abolishment of gel formation.

Penicillin G added to serum produced a marked decrease of relative viscosity, a negative Sia test and abolishment of gel formation.

Penicillin G, 10 million U given intravenously, lowered the serum viscosity by 77 per cent within one hour and increased the previously low sedimentation rates sharply. Rapid rebound of viscosity occurred.

The coincident administration of Benemid with penicillin enhanced this effect and delayed rebound of viscosity considerably.

Addition of cysteamine to serum resulted in dosage-dependent decrease of relative viscosity.

Agar diffusion studies revealed the simultaneous disappearance of the macroglobulin precipitin line and a quantitative increase in the γ-globulin line with DL-penicillamine and cysteamine; no changes were produced by penicillin G.

It is suggested that the compounds studied were responsible for abolishment of cryogel properties and probably produced dissociation of the macroglobulin. Possible clinical implications are mentioned.

ACKNOWLEDGMENT

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INCREASE IN ALDOSTERONE SECRETION BY CAROTID ARTERY CONSTRICTION IN THE DOG AND ITS PREVENTION BY THYROCAROTID ARTERIAL JUNCTION DENERVATION *

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Although it has been shown that the vagus nerves may carry impulses mediating decreases in the rate of aldosterone secretion (1), no equivalent pathway has been demonstrated which might mediate increases in aldosterone secretion. Because of evidence that aldosterone secretion may be increased by some function of a decreased intravascular volume (2), and the evidence cited above that this increase is not dependent upon the integrity of intrathoracic vagal receptors (1), the present experiments were designed to investigate the role of receptors located in the arterial system.

METHODS

Acute experiments were performed on mongrel dogs under pentobarbital anesthesia. The lumboadrenal veins were cannulated (3) and blood samples were collected intermittently for determination of aldosterone by Mills, Casper and Bartter's modification (1) of the method of Neher and Wettstein (4). This method has an absolute sensitivity of 0.1 μg of aldosterone. Losses during extraction and chromatography were determined on every sample by adding C_{14}-cortisone to each specimen before extraction and counting the cortisone region on the final chromatogram after development of the soda fluorescence. The mean recovery rate was 42 ± 9 per cent (SD). The overall sensitivity, considering the amount of the sample taken for analysis and the recovery rate, was 0.5 μg per hour. The final value was corrected for all losses by dividing each observed value by the rate of recovery of that sample. Thus, the only variable altering reproducibility is that of measurement of the soda fluorescence on paper. The technique used was that described by Ayres, Simpson and Tait (5) who found that, in measuring 0.5 μg of aldosterone diacetate, the standard error was 0.01 and the standard errors for measurement of 2, 4, and 8 μg were, respectively, 0.17, 0.43, and 0.73. In experiments in this laboratory, the standard error for measurement of 1 μg of cortisone was found to be 0.04.

Mean and pulse pressures in brachial and lingual arteries and right atrium, and pressures in femoral vein and esophagus, were measured by means of indwelling cannulae and Statham pressure transducers, and recorded continuously on a Sanborn 8-channel recorder. Serial hematocrit determinations were made. In all experiments blood loss was continuously measured, and replaced by transfusion. Blood used for transfusion was obtained from dogs rendered hypervolemic by large saline infusions immediately before bleeding in order to prevent rise in aldosterone-stimulating substances.

Twenty-seven studies were performed on 25 normal dogs, 4 on 3 dogs with carotid arteries stripped of their nerve supply from low in the neck to above the carotid sinuses, 8 on dogs with carotid sinuses denervated but the neck otherwise undisturbed, and 31 on 18 dogs after denervation of the thyrocarotid arterial junctions, with intact carotid sinuses. These operations were performed 3 days to 5 weeks prior to the acute experiments.

Aldosterone secretion was stimulated by supradiaphragmatic inferior vena cava constriction (6) in 29 experiments. In 22 studies the stimulus was bilateral constriction of the common carotid arteries low in the neck, effected by means of chokers consisting of silk loops passed about the arteries and through double tubes sus-

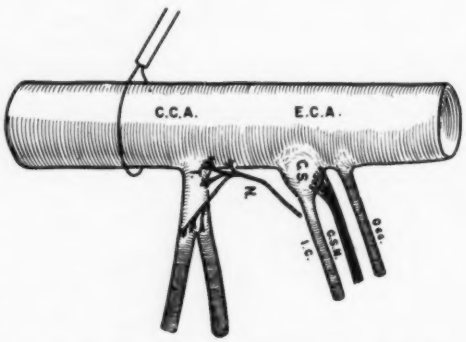

FIG. 1. THE ANATOMY OF THE CAROTID REGION IN THE DOG. Occ. = occipital artery; C.S.N. = carotid sinus nerve; I.C. = internal carotid artery; C.S. = carotid sinus; E.C.A. = external carotid artery; C.C.A. = common carotid artery; N. = nerve arising from thyrocarotid arterial junction; T. = thyrocarotid arterial junction.

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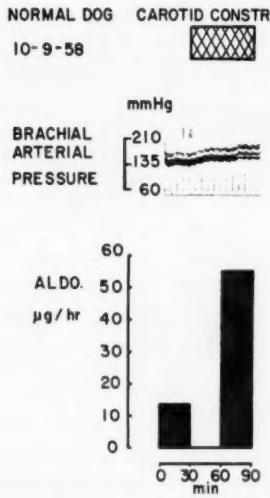

FIG. 2. THE RESPONSE OF BRACHIAL ARTERIAL PRESSURE AND ALDOSTERONE SECRETION TO BILATERAL COMMON CAROTID CONSTRICTION IN A NORMAL DOG.

pended above the arteries. The position of a choker and relevant anatomy are shown diagrammatically in Figure 1.

In each experiment adrenal venous blood was collected to obtain a control value for aldosterone before application of the constriction. All collections were of 30 minute duration. The next collection was begun 30 minutes after constriction. In most experiments, the constriction was then removed, and a sample of blood collected after 1 hour. Following this collection, the constriction was reapplied, and a further sample collected after 30 minutes. In the 90 minutes allowed following release of carotid constriction, aldosterone secretion seldom returned to control levels in normal or in sinus-denervated animals. Data from the second constriction are accordingly excluded from this series (Tables I and III). All specimens from a given experiment were carried through the chromatographic systems together. Each aldosterone value obtained during constriction was referred to its own previous control value for analysis. Each datum thus consisted of a pair of values obtained during control and subsequent constriction.

RESULTS

Effect of carotid constriction in normal dogs. Bilateral constriction of the common carotid arteries in normal dogs initiated the classical Hering reflex (7), the fall in intracarotid pressure giving rise to increased peripheral arterial pressure. In addition, aldosterone secretion was consistently elevated within 60 minutes of constriction. One

TABLE I

*Effect of bilateral common carotid artery constriction on aldosterone secretion, adrenal plasma flow and brachial arterial pressure in intact dogs**

Control			Carotid constriction		
Aldosterone	Adrenal plasma flow	Brachial arterial pressure	Aldosterone	Adrenal plasma flow	Brachial arterial pressure
µg/hr	ml/hr	mm Hg	µg/hr	ml/hr	mm Hg
3.7	196	170/130	7.8	160	195/155
1.9	208	140/120	6.7	234	144/116
20.6	94	176/152	29.4	90	216/184
8.5	58	136/116	7.2	56	160/132
3.9	118	148/120	5.9	144	166/138
4.4	34	174/152	7.8	24	182/150
5.3	102	170/140	10.0	88	172/140
9.0	130	154/122	12.3	88	162/142
4.0	62	166/140	9.9	40	170/142
3.7	76	168/135	8.6	64	177/144
20.0	256	153/123	27.0	202	162/132
13.7	80	153/129	55.3	42	168/148
3.4	72	207/138	17.0	56	210/150
17.0	64	96/84	20.4	86	108/90

* Each value obtained during constriction is shown with its own control, obtained before constriction was begun.

such experiment is shown in Figure 2, and the data for all experiments of this type are shown in Table I and summarized in Figure 5. Carotid constriction thus produced increased aldosterone secretion associated with increased peripheral arterial pressure.

Effects of carotid constriction in dogs with carotid arteries stripped. Constriction of the carotid arteries in these four animals produced neither reflex hypertension nor increase in aldosterone secretion. One such experiment is shown in Fig-

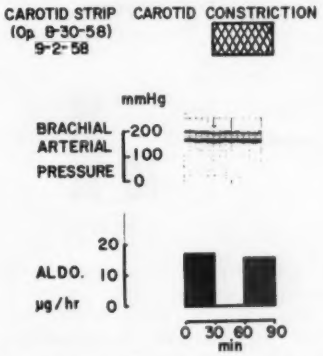

FIG. 3. THE RESPONSE OF BRACHIAL ARTERIAL PRESSURE AND ALDOSTERONE SECRETION TO CAROTID CONSTRICTION IN A DOG WITH BOTH CAROTID ARTERIES STRIPPED OF NERVOUS CONNECTIONS FROM LOW IN THE NECK TO ABOVE THE CAROTID SINUSES.

TABLE II

*Effect of bilateral common carotid artery constriction on aldosterone secretion, adrenal plasma flow and brachial arterial pressure in dogs with both carotid arteries stripped of nervous connections from low in the neck to above the carotid sinuses**

Control			Carotid constriction		
Aldosterone	Adrenal plasma flow	Brachial arterial pressure	Aldosterone	Adrenal plasma flow	Brachial arterial pressure
μg/hr	*ml/hr*	*mm Hg*	*μg/hr*	*ml/hr*	*mm Hg*
16.9	174	216/168	15.5	166	172/140
0(<0.5)	224	153/114	2.3	150	156/129
1.6	124	156/138	1.1	68	153/144
1.0†	42	180/159	2.0	38	177/153

* See footnote, Table I.
† Second constriction.

ure 3, and the data for these experiments are shown in Table II. Stripping the carotid arteries thus abolished both of the responses to carotid constriction seen in the normal animals.

Effects of carotid constriction in dogs with carotid sinuses denervated. Constriction of the carotid artery in these animals produced increase in aldosterone secretion in six out of eight dogs, despite abolition of the Hering reflex by carotid sinus denervation.[1] Thus, no elevation of peripheral pressure, heart rate (or, by inference, cardiac output), accompanied the increases in aldosterone secretion. One such experiment is illustrated in Figure 4, and the data for this group of experiments are shown in Table III and summarized and compared with those from intact animals in Figure 5. There is no significant difference in al-

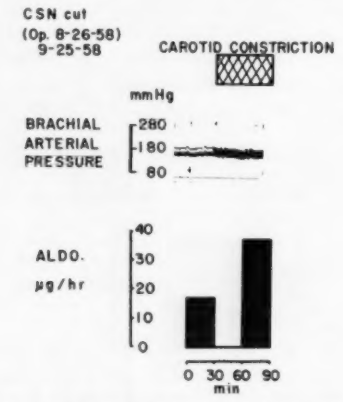

FIG. 4. THE RESPONSE OF BRACHIAL ARTERIAL PRESSURE AND ALDOSTERONE SECRETION TO BILATERAL COMMON CAROTID CONSTRICTION IN A DOG WITH BOTH CAROTID SINUS NERVES CUT. There is a rise in aldosterone in the absence of a reflex rise in brachial arterial pressure (Hering reflex).

[1] The remaining two were early experiments, in which the nerves from the thyrocarotid junctions may have been injured inadvertently.

TABLE III

*Effect of bilateral common carotid artery constriction on aldosterone secretion, adrenal plasma flow and brachial arterial pressure, in dogs with both carotid sinuses denervated**

Control			Carotid constriction		
Aldosterone	Adrenal plasma flow	Brachial arterial pressure	Aldosterone	Adrenal plasma flow	Brachial arterial pressure
μg/hr	*ml/hr*	*mm Hg*	*μg/hr*	*ml/hr*	*mm Hg*
12.7	110	210/189	32.8	83	210/189
7.1	80	134/110	15.1	70	136/112
10.1	156	200/160	4.4	200	206/164
17.0	300	150/126	37.0	208	141/114
7.0	114	196/130	4.7	112	180/140
15.9	80	201/171	25.8	80	192/159
24.2	194	200/172	55.6	258	192/152
5.6	114	150/122	6.4	96	160/134

* See footnote, Table I.

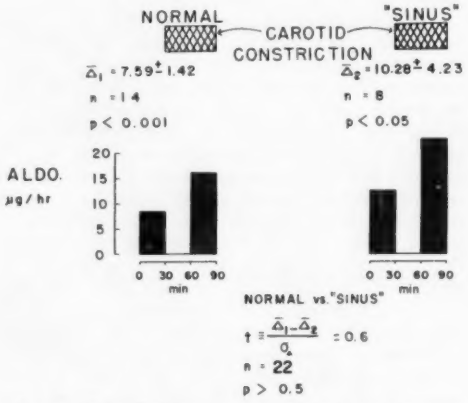

FIG. 5. THE RESPONSE OF ALDOSTERONE SECRETION TO BILATERAL COMMON CAROTID CONSTRICTION IN TWO GROUPS OF EXPERIMENTS. On the left, intact animals. On the right, animals previously sinus-denervated. Above each pair of bars (mean values) is a statistical summary of the data. Below the bars, the two groups are compared by means of the *t* test. Δ = mean change in aldosterone from before to during constriction; n = number of pairs of values (control and during constriction). A highly significant rise in aldosterone secretion is associated with carotid constriction, and there is no significant difference between the normal and sinus-denervated groups.

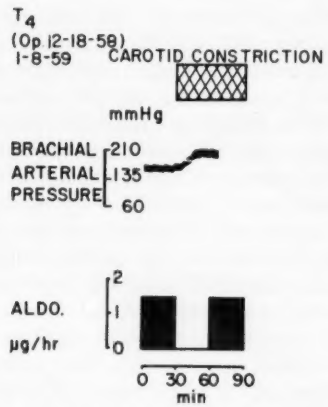

FIG. 6. THE RESPONSE OF BRACHIAL ARTERIAL PRESSURE AND ALDOSTERONE SECRETION TO BILATERAL COMMON CAROTID CONSTRICTION IN A DOG WITH BOTH THYROCAROTID ARTERIAL JUNCTIONS PREVIOUSLY DENERVATED. There is no rise in aldosterone secretion but an intact Hering reflex.

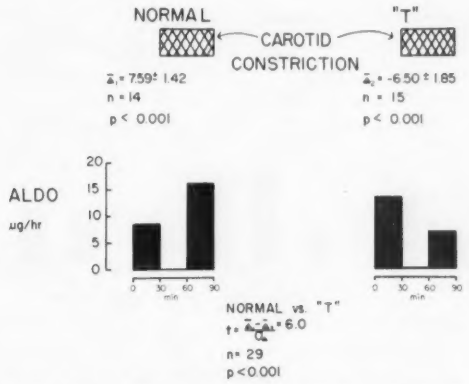

FIG. 7. THE RESPONSE OF ALDOSTERONE SECRETION TO CAROTID CONSTRICTION IN TWO GROUPS OF EXPERIMENTS. On the left, intact animals. On the right, animals with previous denervation of both thyrocarotid arterial junctions. The data are arranged as in Figure 5. The fall in aldosterone is highly significant in the group on the right, and the difference between the two groups is highly significant.

dosterone secretion in response to carotid constriction between sinus-denervated and intact animals.

Effects of carotid constriction in dogs with thyrocarotid junction denervation. Constriction of the carotid arteries in dogs in which both thyrocarotid arterial junctions had been previously denervated produced no increase in aldosterone secretion despite the presence of intact Hering reflexes. Indeed, in most of these experiments, aldosterone secretion actually fell. One such experiment is shown in Figure 6, and the data for this group of experiments are shown in Table IV

TABLE IV

*Effect of bilateral common carotid artery constriction on aldosterone secretion, adrenal plasma flow and brachial arterial pressure, in dogs with denervation of both thyrocarotid arterial junctions**

Control			Carotid constriction		
Aldosterone	Adrenal plasma flow	Brachial arterial pressure	Aldosterone	Adrenal plasma flow	Brachial arterial pressure
µg/hr	ml/hr	mm Hg	µg/hr	ml/hr	mm Hg
8.5	40	141/114	3.8	40	168/141
1.1	26	96/81	1.7	22	111/96
8.8	130	153/129	4.4	72	175/141
16.0†	54	156/138	11.2	40	216/180
35.7	90	141/108	8.5	66	156/132
1.4†	48	174/153	1.4	40	222/198
15.9	248	150/96	6.1	174	156/114
6.9†	116	156/132	5.3	114	168/141
11.3	98	164/144	11.0	80	186/164
8.1	108	140/104	5.7	110	176/144
6.1	54	140/116	6.6	88	148/108
33.5	76	159/129	19.0	58	186/156
12.7	78	162/122	13.2	70	177/144
3.1†	42	156/124	1.8	112	180/144
38.0†	66	174/141	30.0	44	195/168

* See footnote, Table I.
† Second constriction.

TABLE V

*Effect of supradiaphragmatic inferior vena cava constriction on aldosterone secretion**

Intact dogs		Dogs with both thyrocarotid arterial junctions denervated	
Control	Constricted	Control	Constricted
µg/hr		µg/hr	
1.8	8.0	10.0	8.5
3.3†	13.6	6.0†	3.0
3.1	7.6	0(<0.5)	4.3
2.5	7.2	9.6†	16.0
6.9	11.4	1.1	0.6
3.4†	7.9	2.0†	3.2
2.6	4.3	5.4	0.6
2.5	7.2	3.5	7.3
1.2	4.3	2.9†	14.7
3.0	10.4	8.5†	8.6
5.8	9.5	7.1	4.5
1.3	6.0	7.7†	6.1
5.3	20.0	6.6	1.5
		7.2†	5.1
		2.9	2.5
		1.5†	1.4

* See footnote, Table I.
† Second constriction.

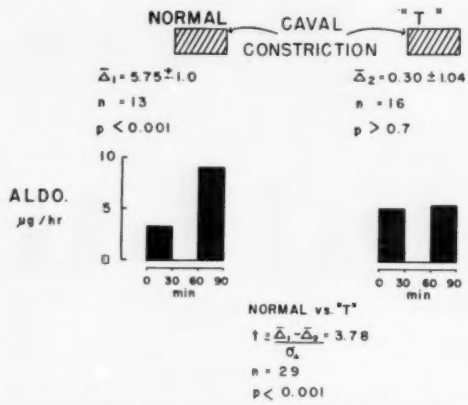

FIG. 8. THE RESPONSE OF ALDOSTERONE SECRETION TO CONSTRICTION OF THE INFERIOR VENA CAVA IN TWO GROUPS OF EXPERIMENTS. On the left, intact animals. On the right, animals with previous denervation of both thyrocarotid arterial junctions. The data are arranged as in Figure 5. The difference between the two groups is highly significant.

and summarized and compared with those from intact animals in Figure 7. Thus, thyrocarotid arterial junction denervation prevented the rise in aldosterone secretion following carotid constriction without abolishing the rise in peripheral arterial pressure.

Effects of caval constriction in normal dogs and in dogs with thyrocarotid arterial junction denervation. Constriction of the supradiaphragmatic inferior vena cava is a well documented stimulus to increase of aldosterone secretion (1, 6). The results of this procedure in 11 normal dogs are shown in Table V. In dogs in which the thyrocarotid arterial junctions had been previously denervated, an exactly comparable degree of caval constriction had no consistent effect on the secretion of aldosterone. The results of this procedure in 16 experiments are shown in Table V. The data are summarized and compared in Figure 8.

DISCUSSION

These data suggest a dual mechanism regulating the secretion of aldosterone, whereby stimuli mediating increases are conveyed to the central nervous system via the nerves originating at the thyrocarotid arterial junctions, while those mediating decreases are conveyed via the vagus nerves. It has been shown previously (1) that vagotomy alone does not produce an increase in the rate of aldosterone secretion. Carotid constriction, on the other hand, is evidently an adequate stimulus to increase of aldosterone secretion. This increase is independent of pressure changes in the peripheral arterial (extracarotid) system. This is clearly demonstrated by the continued effectiveness of the stimulus after carotid sinus denervation as well as by the ineffectiveness of the stimulus, despite intact pressure response, in animals with thyrocarotid arterial junction denervation.[2]

It has previously been suggested that aldosterone secretion is regulated by some function of extracellular (8) or intravascular (2) fluid volume. The present data suggest that the significant parameter is some function of intracarotid blood volume. The data presented here do not indicate what that effective parameter is, but evidence drawn fram these and other experiments (9) suggests that it is primarily a decrease in intracarotid pulse pressure which leads to the increase in aldosterone secretion.

Farrell (10) has suggested that aldosterone secretion is regulated principally by means of intracranial receptors, primarily measuring sodium concentration. Evidence from experiments in man, on the other hand, suggests that neither serum sodium concentration nor total body sodium can control aldosterone secretion, which can be made to vary independently of both (8). The present data, in addition, provide a mechanism whereby the central nervous system may receive afferent impulses from the periphery leading to increase or decrease in aldosterone secretion. Thus, it is no longer necessary to postulate the existence of central receptors. However, the existence of such receptors is not excluded by these experiments which, in particular, shed no light on the mechanism by which changes in body potassium may modify aldosterone secretion.

If, as is suggested by these experiments, there is central integration of afferent impulses modifying aldosterone secretion, then it is possible that the central nervous system releases a hormone

[2] Table V includes four pairs of values (from two dogs) in which aldosterone secretion rose with constriction. It seems likely, in retrospect, that denervation was not complete in these animals. The data were not excluded from analysis.

which regulates adrenal secretion of aldosterone and constitutes the efferent limb of this reflex, as was previously suggested by Rauschkolb and Farrell (11). Evidence for the existence of such a hormone has been supplied by both Denton, Goding and Wright (12) and Yankopoulos, Davis, Kliman and Peterson (13). [In the former experiments, however, aldosterone was not measured directly, but was estimated from changes in salivary Na : K ratios. On the other hand, there is evidence (14, 15) that adrenal isolation is not complete in the preparation of Hilton and co-workers (16) used in the latter experiments. Accordingly, blood pressure or pharmacological changes in the recipient animal could themselves be adequate stimuli to increase in aldosterone secretion. Hemodynamic changes and norepinephrine (17) have both been shown to alter baroceptor activity, which, in turn, may modify aldosterone secretion (9). Furthermore, cross circulation itself results in maximal secretion of Porter-Silber steroids (18). Under this circumstance, further increase of ACTH secretion may cause a rise in aldosterone secretion without changing corticosterone secretion (19). Thus, increase in the aldosterone : corticosterone ratio is not in itself sufficient evidence of a stimulating substance other than ACTH.] Although it seems likely that a neurohormone is active in the control of aldosterone secretion, its existence has not as yet been adequately demonstrated.

In these experiments, it has clearly been possible to measure rises in aldosterone secretion. In the preparation used by Farrell and associates, this was not possible, as all experimental animals secreted at maximal rates (10, 20), presumably as a result of the blood loss which is an integral part of that method. Accordingly, control data were derived from other animals. In the present experiments, blood was replaced as it was lost, and each animal could serve as its own control.

The apparent difference between the effects of carotid and caval constrictions in dogs with previous thyrocarotid arterial junction denervation, in which the first group showed lowering of aldosterone secretion ($p < 0.001$), while the second showed no change in aldosterone secretion, is not well explained. However, carotid constriction causes a reflex increase in right atrial pulse pressure (21), while atrial pressure is lowered with caval constriction. It is possible that this increase in pressure activates the "decrease" mechanism mediated by the vagus nerves, thus accounting for the lowering of aldosterone with carotid constriction in the presence of thyrocarotid denervation.

SUMMARY

Constriction of the common carotid arteries low in the neck produces increased secretion of aldosterone. This effect is independent of changes in peripheral arterial pressure, but is abolished by bilateral denervation of the thyrocarotid arterial junctions. The rise in aldosterone normally seen with constriction of the inferior vena cava is also prevented by denervation of this region. It is suggested that aldosterone secretion is regulated by a dual mechanism, in which decreases in secretion are mediated by the vagus nerves, while increases in secretion are mediated primarily by receptors located at the thyrocarotid arterial junction. Aldosterone secretion may thus be increased by local decrease in intracarotid volume, independent of total extracellular or even intravascular volumes.

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THE BIOSYNTHESIS AND TURNOVER OF OXALATE IN NORMAL AND HYPEROXALURIC SUBJECTS*

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Oxalate is a normal constituent of human urine. Since oxalate is found in many foods it has frequently been assumed that urinary oxalate is largely, if not entirely, of dietary origin. However, animal studies have shown that oxalate may arise endogenously, and glycine, glyoxylic acid, glycolic acid (3–5) and ascorbic acid (6, 7) have been shown to be precursors of urinary or tissue oxalate under various circumstances.

The origin of urinary oxalate has long been an important physiological problem because of the high incidence of calcium oxalate urolithiasis in many parts of the world. Recently, interest in the biosynthesis of oxalate in man has been greatly stimulated by recognition of the syndrome of primary hyperoxaluria and oxalosis, a disorder in which excessive production of oxalate occurs and leads to deposition of calcium oxalate crystals in renal parenchyma and extrarenal tissues. Ascorbic acid has previously been shown to be a precursor of some urinary oxalate of normal man (6, 8). In this paper glycine is shown to be a major precursor of urinary oxalate of normal and hyperoxaluric subjects. In one subject with primary hyperoxaluria, glycine incorporation into urinary oxalate was excessive, whereas in another subject with an inconstant hyperoxaluria, glycine incorporation was not abnormal. Measurements of the miscible pool of oxalate and of the rate of its turnover have been made by conventional isotopic dilution techniques, and a gross expansion of the miscible pool of oxalate demonstrated in a hyperoxaluric subject.

EXPERIMENTAL METHODS

Materials. Glycine 1-C^{14}, specific activity 2.2 mc per mmole, and oxalic acid 1,2-C^{14}, specific activity 2.6 mc per mmole, both of radiopurity > 99 per cent, were purchased from Isotopes Specialties Co. Burbank, Calif.

Control subjects. Control subjects were hospitalized patients without known metabolic, gastrointestinal or genitourinary disease, in whom routine tests [urinalysis, blood urea nitrogen (BUN), phenolsulfonphthalein (PSP) excretion] disclosed no evidence of renal disease. They were permitted a regular hospital diet except that foods of high oxalate content [cocoa, chocolate, tea, rhubarb, beets and spinach (9)] were withheld for a few days before and during the study period. The basal excretion of oxalate [as $(COOH)_2 \cdot 2H_2O$] ranged from 11.6 to 36.0 mg per day in the control subjects.

Hyperoxaluric subjects. C.B. was a 30 year old male who was first seen at Duke Hospital in 1950 at the age of 23. He had had recurrent urolithiasis since age 10 and had required pyelolithotomy at ages 18 and 19. On admission in 1950 nephrolithotomy and pyelolithotomy were again performed, urinary tract infection was present and the stones were found to contain calcium, oxalate, phosphate and small amounts of carbonate. Subsequent to 1950 he required litholapaxy on several occasions. He passed gravel almost daily, and had severe renal colic on numerous occasions each month. Repeated analyses of serum calcium, phosphorus and alkaline phosphatase were normal. Mild elevations of nonprotein nitrogen (NPN) (maximum, 60 mg per 100 ml), serum uric acid (maximum, 6.2 mg per 100 ml) and cholesterol (268 mg per 100 ml) were noted, and PSP excretion tests ranged from 41 to 45 per cent in 2 hours. Urine cultures revealed organisms of the Klebsiella and Aerobacter groups, and he was treated with various agents without success.

In 1958 he was found to be excreting abnormal quantities of oxalate, ranging from 103 to 143 mg per day. He was therefore admitted for further study. General physical examination was normal except for well healed surgical scars. Blood pressure was 114/80. Urinalysis revealed pH 6.5, specific gravity 1.011, a trace of protein, and innumerable leukocytes in the sediment. NPN was 48, serum uric acid 6.8, calcium 9.3, and phosphorus 3.5 mg per 100 ml. X-ray revealed bilateral staghorn calculi but no evidence of nephrocalcinosis. He was placed on a diet calculated to contain 62 mg of oxalate daily during the study period.

A familial history of urolithiasis was obtained, and a sister was found also to have a slightly elevated urinary excretion of oxalate (65 mg per day). This sister has passed stones composed of calcium oxalate; both parents and a paternal cousin have also passed stones. The parents and a brother of the patient died of nonrenal disorders.

N.M., a 48 year old female with a 25 year history of recurrent calcium oxalate urolithiasis, was studied 4

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months following left nephrectomy. The kidney was nonfunctioning and showed chronic pyelonephritis and hydronephrosis but no nephrocalcinosis or parenchymal oxalate deposits. One week after operation her urinary oxalate excretion was 117 mg per 24 hours; 4 months later when she was admitted for further studies it was initially 90 mg per 24 hours. She was placed on a standard diet calculated to contain each day: protein 30.4 g, calcium 112.5 mg, ascorbic acid 33 mg, pyridoxine 0.5 mg. By analysis it contained 129 mg of oxalic acid dihydrate per day. After 6 days on this diet her urinary calcium excretion was 400 to 600 mg per day. Serum calcium was 9.1 mg per 100 ml, phosphorus 4.2 mg per 100 ml and alkaline phosphatase 2.6 U per 100 ml. Studies to date have not established the cause of her hypercalcuria. Because of a decline in hyperoxaluria while on the experimental diet, she remained on a 30 g protein regimen following the study. Urinary oxalate excretion has remained below 40 mg per day on 3 different examinations over a 5-month period. Her urine sediment is now free from calcium oxalate crystals, which were formerly present in abundance in all specimens.

Tracer studies. Glycine 1-C^{14} was administered orally to subjects in the fasting state in 25 or 50 μc doses (11.4 or 22.7 μg) in 100 ml of water. Oxalic acid 1,2-C^{14} was administered slowly intravenously in 1 μc doses (0.05 mg) in 2 ml of sterile (Seitz-filtered) solution in water. Both compounds were administered after an overnight fast.

In two subjects oxalate and hippurate were isolated simultaneously, from successive 2-hour urine samples. Two hours after ingestion of labeled glycine, and at 4-hour intervals for 3 additional doses, the subjects ingested 3 g of sodium benzoate, so as to insure a large urinary output of hippuric acid.

Urine specimens were collected in clean glass jugs containing 5 ml of concentrated HCl. Respiratory CO_2 was collected in carbonate-free, 1 N sodium hydroxide and precipitated as barium carbonate.

All counting was done in stainless steel planchets in a Robinson gas-flow counter (10). Calcium oxalate samples varied in mass from 4 to 36 mg and were corrected for self-absorption to a standard mass of 22 mg per planchet (1.54 sq cm). Barium carbonate samples were counted in infinite thickness. Hippuric acid samples weighed from 5 to 15 mg and were corrected for self-absorption to a standard mass of 3.3 mg per planchet. Appropriate conversion factors were employed for comparison of counting values of glycine, oxalic acid, calcium oxalate and hippuric acid when required.

Isolation of oxalate from urine. Each urine sample was filtered through Whatman no. 42 paper in a Buchner funnel if any cloudiness or precipitate was grossly visible, and aliquots were taken for determination of oxalate by the method of Archer, Dormer, Scowen and Watts (11). Thirty mg of oxalic acid dihydrate was added to the remainder of each urine specimen as carrier and the volume reduced to approximately 20 ml under reduced pressure at 30° C. The pH was adjusted to 5.5 with 8 N ammonium hydroxide, and 5 ml of 5 per cent $CaCl_2$ was then added. The samples were stored at 4° C overnight, centrifuged, and the supernatant solution discarded.

Calcium oxalate was then purified by a slight modification of the method of Burns, Burch and King (12). The precipitate was washed 2 or 3 times with 4 ml of 0.35 N NH_4OH, to remove colored impurities, and dissolved in 6 ml of 1 N sulfuric acid; after centrifugation the residue was discarded. The supernatant was placed in a glass microextractor [1] and extracted continuously with 100 ml of ether for at least 6 hours. At the end of the extraction period 10 ml of distilled water was added to the flask and the ether evaporated on a steam bath. The aqueous phase was transferred to a 40 ml conical centrifuge tube and the pH was adjusted to 5.5 with 8 N ammonium hydroxide. Calcium oxalate was reprecipitated by addition of 5 ml of 5 per cent $CaCl_2$. The sample was stored overnight in the cold, centrifuged, and the supernatant discarded. The precipitate was dissolved in 1 N HCl, treated with Darco, filtered, and recrystallized by adjustment of the pH to 5.5 and addition of 5 ml of 5 per cent $CaCl_2$. The last procedure was repeated several times.

The samples were dried at 80° C in a vacuum oven, ground with a stout glass rod in acetone, and transferred by pipet to a tared stainless steel planchet for counting.

Isolation of hippuric acid from urine. Urinary hippuric acid was isolated according to Quick (13) and purified by treatment with Darco and by repeated recrystallizations from hot water (14). The melting points of the isolated samples were identical with those of authentic hippuric acid.

Calculations. The standard isotopic dilution equation for calculating the miscible pool of oxalate is as follows (15): $A = a\ (I_1/I_o - 1)$, where A is the size of the miscible pool, a is the quantity of labeled material added to the pool, I_1 is the specific activity of the injected material, and I_o is the theoretical specific activity in the miscible pool at the moment of mixing. In the present study this equation was simplified, and the pool was calculated as follows: $A = (aI_1)'/I_o$. In this equation $(aI_1)'$ represents the total counts administered minus the labeled oxalate which was excreted too rapidly to have mixed thoroughly within the pool (see below). The term "-1" of the first equation may be ignored, since "a" was very small (0.05 mg) compared with values of A (4 to 8 mg). The intercept, I_o, and the slope, $-K$, and their standard deviations, were calculated by the method of least squares. The biological half-time, $T_{\frac{1}{2}}$, was obtained from the expression (15) $K = \log 2/T_{\frac{1}{2}}$. The standard deviation of A was calculated as previously described (16).

RESULTS

Oxalate excretion

The urinary excretion of oxalate in 18 normal subjects (37 samples) on a mixed diet ranged from 6.3 to 44.8 mg, and averaged 22.1 mg per 24 hours, [as oxalic acid dihydrate, $(COOH)_2 \cdot 2H_2O$]. No consistent diurnal variation was found in two

[1] Ace Glass Co., Inc., Vineland, N. J., Catalog no. 6820.

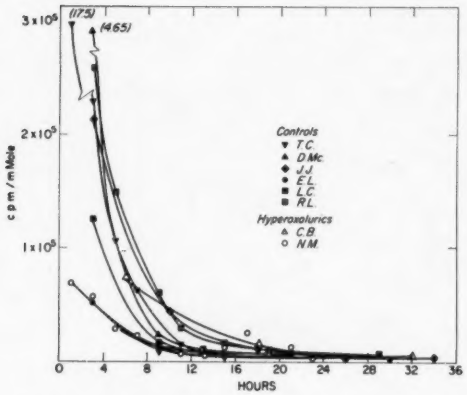

FIG. 1. INCORPORATION OF GLYCINE 1-C^{14} INTO URINARY OXALATE IN CONTROL AND HYPEROXALURIC SUBJECTS The data have been normalized to a standard dose of 25 μc of C^{14}, given orally as carrier-free glycine 1-C^{14}.

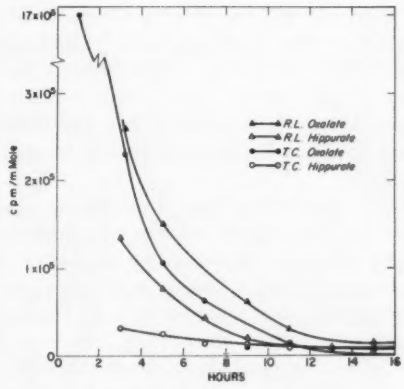

FIG. 3. COMPARISON OF ENRICHMENT OF URINARY OXALATE AND HIPPURATE IN TWO CONTROL SUBJECTS FOLLOWING INGESTION OF 25 μC OF GLYCINE 1-C^{14}. Sodium benzoate, 3 g, was given orally at 2, 6, 10 and 14 hours to insure a large urinary output of hippurate.

subjects in each of whom 12 consecutive 8-hour collections were obtained, nor was urinary oxalate excretion significantly correlated with urine volume ($R = 0.87, 0.68$; $p = 0.06, 0.20$).

Glycine incorporation

Urinary oxalate became highly labeled within a few hours following oral administration of 25 μc of glycine 1-C^{14} in each of six control subjects (Figure 1). The initial (3-hour) specific activity values ranged from 49,000 to 450,000 cpm per mmole. This ninefold range was found in subjects having only a threefold range in urinary oxalate values; specific activity values were not inversely correlated with excretion values. Enrichment values declined rapidly so that, by 24 to 30 hours, the counting values of urinary oxalate approached background.

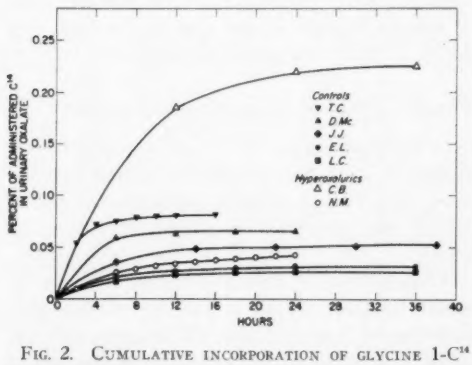

FIG. 2. CUMULATIVE INCORPORATION OF GLYCINE 1-C^{14} INTO URINARY OXALATE IN CONTROL AND HYPEROXALURIC SUBJECTS. Data are expressed in percentage of administered C^{14}.

The cumulative incorporation of C^{14} into urinary oxalate (Figure 2) ranged from 0.027 to 0.081 per cent (mean, 0.051 per cent) of the administered dose of glycine 1-C^{14} in five control subjects.

In two control subjects who received load doses of sodium benzoate beginning 2 hours after ingestion of glycine (Figure 3), urinary oxalate was significantly more highly labeled than was urinary hippurate between 2 and 10 hours. Both substances exhibited typical die-away enrichment curves in both subjects.

These studies establish that glycine serves as a precursor of at least some of the urinary oxalate in normal man, and suggest *1*) that relatively little dilution occurs between glycine and oxalate, *2*) that the miscible pool of oxalate is small, and *3*) that the turnover of the oxalate pool is rapid. Measurements of the miscible pool of oxalate and of its turnover were then attempted by standard isotopic dilution techniques.

The miscible pool of oxalate and the rate of its turnover

When 50 μg of oxalic acid 1,2-C^{14} was injected intravenously into control subjects, a portion rap-

idly appeared in urine. A semilogarithmic plot of specific activity of urinary oxalate against time showed that a linear decay was achieved after 4 hours (Figures 4, 5). After about 18 hours the specific activity values were so low that their accuracy was doubtful, and their usefulness small in this study.

By the time that mixing of the labeled oxalate within the oxalate pool was complete, presumably about 4 hours after its administration, a large fraction of the test dose of labeled oxalate had already been excreted. Since eventually 89 to 99 per cent of the administered tracer was recovered unchanged in urine (see below), nonrenal loss of oxalate was not an appreciable factor, and a correction for the labeled oxalate which had failed to mix adequately could be applied on the basis of the 0 to 4 hour urinary fraction. The regression of the linear portion of the specific activity-time curve was calculated by the method of least squares, omitting data of 0 to 2 and 2 to 4 hour points. In each case the zero intercept was a highly significant value, $p < 0.01$. The oxalate-C^{14} excreted in excess of that anticipated from the extrapolated curve was then subtracted from the administered dose (aI_1) and further calculations were made on the basis of the "corrected administered dose" $(aI_1)'$. The subtrahend averaged about 60 per cent of (aI_1), and therefore the measurements of oxalate and of specific activity in the initial samples are critical to this correction.

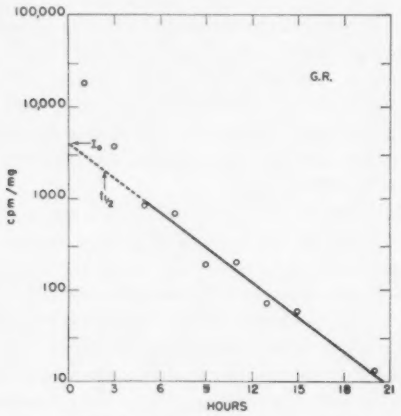

FIG. 4. SPECIFIC ACTIVITY OF URINARY OXALATE FOLLOWING INTRAVENOUS ADMINISTRATION OF OXALIC ACID 1,2-C^{14} TO A CONTROL SUBJECT. The data of this experiment (Subject G.R., Table I), when analyzed by the method of least squares, indicated a miscible pool of 4.9 ± 0.7 mg, and a turnover rate of 1.4 ± 0.3 mg per hour.

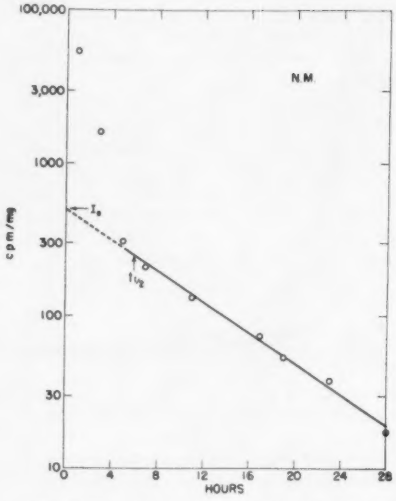

FIG. 5. SPECIFIC ACTIVITY OF URINARY OXALATE FOLLOWING INTRAVENOUS ADMINISTRATION OF OXALIC ACID 1,2-C^{14} TO A HYPEROXALURIC SUBJECT. These data (Subject N. M., Table I) indicated a miscible pool of 39.1 ± 2.4 mg and a turnover rate of 4.6 ± 0.3 mg per hour.

Table I shows that the calculated miscible pool of oxalate (expressed as oxalic acid dihydrate) ranged from 4.9 ± 0.7 to 8.6 ± 1.1 mg in three control subjects. The biological half-time of the pool ranged from 2.2 ± 0.3 to 2.8 ± 0.3 hours. The calculated daily turnover of oxalate in these three control subjects ranged from 34.1 ± 6.7 to 63.8 ± 11.3 mg per day. In each case the value of urinary oxalate on the day of the study fell within 2 SD of the calculated turnover.

Physiological disposition of oxalate in man

In three studies on control subjects and in two studies on N.M., a hyperoxaluric subject (see below), from 89 to 99 per cent of the injected oxalate 1,2-C^{14} was recovered unchanged in urine in 36 hours.

Respiratory CO_2 was collected at frequent intervals for 6 hours from two control subjects who had received 1 μc of oxalate-C^{14} intravenously and orally, respectively. In both cases all samples of CO_2, counted as barium carbonate, were devoid

TABLE I

Miscible pool of oxalate and its turnover

Subject	Age	Wt. (W)	Dose injected (aI$_0$)	Excreted before mixing	Intercept (I$_0$)	Miscible pool (A)	Turnover			Urinary oxalic acid	Dose recovered, in 36 hrs
							$T_{1/2}$	(K)	(KA)	(KA×24)	
		kg	*cpm*	*cpm*	*cpm/mg*	*mg*	*hrs*	*pool/hr*	*mg/hr*	*mg/day*	*mg/day*	%
L.H.	39	85	84,500	44,948±7,403*	3,769±930	8.0±0.5	2.8±0.3	0.25±0.02	2.0±0.3	48.0±5.0	38.6	89
G.R.	38	70	84,500	59,336±3,751	4,010±1,173	4.9±0.7	2.4±0.3	0.29±0.04	1.4±0.3	34.1±6.7	37.1	97
E.T.	51	85	84,500	49,883±3,353	3,123±702	8.6±1.1	2.2±0.3	0.31±0.04	2.7±0.5	63.8±11.3	44.8	95
N.M.†	48	62	80,464	54,372±544	503±42	39.1±2.4	6.0±0.2	0.12±0.00	4.6±0.3	109.7±6.7	80.6	90
N.M.†	48	62	80,464	52,288±3,877	9,533±3,628	1.8±1.0	4.5±0.5	0.16±0.02	0.3±0.2	8.2±3.8	12.3	99

* Mean ±1 SD given throughout this table.
† Second study performed 13 days after first; see text.

of enrichment by the counting method employed, which would have detected as little as 0.01 per cent of the administered dose in any single sample.

Studies on patients with hyperoxaluria and recurrent calcium oxalate urolithiasis

Subject C. B. The male subject with probable primary hyperoxaluria was given an oral dose of 25 μc glycine 1-C^{14}, and incorporation of C^{14} into urinary oxalate was demonstrated. The specific activity values were comparable with those found in control subjects at equivalent points in time (Figure 1); however, a cumulative incorporation of 0.22 per cent of the administered dose was found (Figure 2), a value some fourfold greater than the mean cumulative incorporation value found in the control subjects.

Subject N.M. During the first week on the special diet the urinary oxalate excretion of N.M., the female hyperoxaluric subject, progressively declined, and after the seventh day it remained within the normal range. The factors involved in the disappearance of hyperoxaluria in this patient are not understood and are the subject of continuing studies which will be reported elsewhere. The studies with glycine 1-C^{14} and oxalic acid, 1,2-C^{14} will be of interest to record here.

Following oral administration of 25 μc of glycine 1-C^{14}, her urinary oxalate was maximally labeled in the initial (0 to 2 hour) specimen. The specific activity values were similar to those found in E.L., who showed the lowest enrichment of the six control subjects (Figure 1). After 30 hours, urinary oxalate contained only trivial quantities of isotope. The cumulative incorporation of C^{14} into urinary oxalate was 0.047 per cent, a value close to the mean of those obtained in the control subjects (Figure 2).

The miscible pool and the rate of its turnover were determined twice on this patient. On the second day of the study her pool was 39.1 ± 2.4 mg and its turnover 109.7 ± 6.7 mg per day (Figure 5). On this day her urinary oxalate excretion was 80.6 mg per 24 hours. Subsequently, when her hyperoxaluria had disappeared, her miscible pool was found to be 1.8 ± 1.0 mg and its turnover 8.2 ± 3.8 mg per day. Her urinary oxalate excretion on the day of this study (Day 14) was 12.3 mg per 24 hours.

DISCUSSION

Physiological disposition of oxalate. Dakin (17) suggested in 1922 that oxalate is a nonessential end product not further metabolized in mammalian tissue. Subsequent studies in animals (4, 18, 19) and the present studies in man have shown that labeled oxalate is not significantly converted to respiratory CO_2, and that an intravenous tracer of labeled oxalate is almost quantitatively excreted in urine.

Biosynthesis of oxalate. Ratner, Nocita and Green (3), Weinhouse and Friedmann (4), and Nakada and Weinhouse (5) have shown in animal studies *in vivo* and *in vitro* that glycine may be converted to oxalic acid, via glyoxylic acid. Glycine is converted to glyoxylic acid by glycine oxidase, and possibly as a by-product of transamination with α-ketoglutaric acid, a reaction whose equilibrium, however, lies far toward glycine. Glyoxylic acid is converted to oxalic acid by enzymes which are less well understood. One may be xanthine (aldehyde) oxidase (20); another, possibly a mutase, also exists (3). Glyoxylic acid is the only known direct precursor of oxalic acid in mammalian systems. Glycolic acid is readily converted to glyoxylic acid by a flavoprotein, glycolic oxidase (21), and sparingly by lactic dehydrogenase and DPN (5). The pathway by which ascorbic acid (4, 16) contributes carbon atoms to oxalic acid is not known. In bacterial systems (22), oxalosuccinic acid, oxaloacetic acid and β-ketoadipic acid also serve as precursors of oxalic acid.

In the control subjects, 0.03 to 0.08 per cent of glycine 1-C^{14} was converted to oxalic acid. The normal urinary excretion of oxalic acid (mean 22 mg) approximates 0.03 to 0.08 per cent of the turnover of the first metabolic glycine pool in man, estimated to be about 0.5 to 1 g per kg per day (23, 24), or 35 to 70 g per 70 kg man per day. Thus, glycine is at least a major precursor of urinary oxalate in normal persons, but the apparent correspondence of values given above should not be stressed unduly, for there is also evidence that an appreciable percentage of urinary oxalate comes from precursors other than glycine. Crawhall, Scowen, de Mowbray and Watts (25) studied a single control subject with glycine 1-C^{13}, and concluded that at least half of the urinary oxalate was not derived from glycine in this subject.

In the present studies urinary oxalate was labeled considerably in excess of urinary hippurate. When no sodium benzoate is administered, urinary hippurate and urinary free glycine are comparably labeled within a few hours following administration of glycine N^{15} or C^{13} (25). The work of Gray and Neuberger (26) and of Arnstein and Neuberger (27) suggests that large doses of benzoate stimulate synthesis *de novo* of new glycine, or mobilize glycine from pools not ordinarily in rapid equilibrium with the pool from which hippurate derives its glycine. Benzoate loading probably explains why hippurate glycine was less highly labeled than oxalate in the present studies, whereas in the studies of Crawhall and associates (25) free glycine (and presumably hippurate also) was more highly labeled than was oxalate. Finally, the studies reported herein offer additional evidence for the existence of multiple metabolic pools of glycine in poor equilibrium one with another, as previously suggested by studies of a number of other workers (14, 27, 28).

The miscible pool of oxalate and its biological turnover. The isotopic dilution technique for determination of the pool and turnover of a substance is based upon a number of assumptions and is subject to limitations which have been discussed in detail previously (29). In the present study the major limitations are two: *1*) the relatively large fraction of the injected tracer which is rapidly excreted in urine, and *2*) the small quantity of oxalate present in 2-or 4-hour urine specimens. The first problem has been approached by eliminating from calculations the labeled oxalate which was excreted without mixing with the pool. The second problem was minimized by employing the average of replicate analyses.

The values shown in Table I for the miscible pool, biological half-time and turnover of oxalate in the body are in reasonably close agreement in three control subjects. The turnover values are in satisfactory agreement with urinary excretion data, although the turnover values tended to be a little larger, both in controls and in the hyperoxaluric subject. Animal studies suggest that some endogenous oxalate is excreted in the feces, but the urinary recovery of labeled oxalate of 94 per cent suggests that nonrenal excretion of en-

dogenous oxalate is not a large fraction in normal subjects. Since only 90 per cent of oxalate C^{14} was recovered in N.M. when her oxalate pool was large, whereas 99 per cent was recovered when it was small, the possibility remains that a larger fractional nonrenal disposal of oxalate occurs in the presence of expanded body stores of oxalate.

Hyperoxaluria. In C.B., the incorporation of glycine 1-C^{14} into urinary oxalate was 0.22 per cent, a value about fourfold greater than the mean control value. This patient's urinary oxalate excretion was four- to fivefold greater than the mean normal value. Thus it appears that about the same percentage of urinary oxalate was derived from glycine in C.B. as in controls. Crawhall, Scowen and Watts (28) reached a similar conclusion in two hyperoxaluric subjects given glycine 1-C^{13}. These results, considered together with evidence *in vitro* that oxalate synthesis from glycine becomes appreciable only when the glyoxylic acid pool is greatly expanded, suggest that a block in glyoxylic acid metabolism may exist in primary hyperoxaluria, possibly of glyoxylic-glutamic transaminase (30) or of glyoxylic dehydrogenase (31). Either block (Figure 6) could account for overproduction of oxalate from more than one precursor of its obligatory intermediary, glyoxylic acid (32).

In N.M. the incorporation study was performed at a time when the miscible pool and the daily excretion of oxalate were declining and approaching normal values (Day 5). The metabolic process which had led to hyperoxaluria had inadvertently been corrected, and the possibility that oxalate had previously been overproduced from glycine is not excluded. What relationship, if any, the hyperoxaluria demonstrated in this subject bears to that found in patients with primary hyperoxaluria and oxalosis is obscure at present. Secondary hyperoxaluria is known to occur in man in sarcoid and hepatic cirrhosis (33) and in animals which are deficient in pyridoxine (34). Additional studies are required to elucidate the nature of the metabolic or absorptive defect responsible for the inconstant hyperoxaluria in this subject.

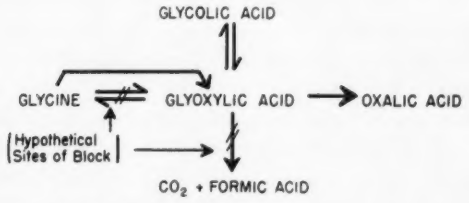

FIG. 6. POSSIBLE SITES OF HYPOTHETICAL BLOCK IN PRIMARY HYPEROXALURIA.

SUMMARY

1. In five control subjects, from 0.027 to 0.081 per cent of a tracer dose of glycine 1-C^{14} appeared in urinary oxalate, apparently by a fairly direct biosynthetic route. The sequence, glycine⟶ glyoxylate⟶ oxalate, is known in animals.

2. The miscible pool of oxalate ranged from 4.9 to 8.6 mg in three control subjects; its turnover ranged from 34 to 63 mg per day. The latter values agreed satisfactorily with excretion values. Labeled oxalate was not catabolized to CO_2, and was recovered almost quantitatively (89 to 99 per cent) in urine.

3. In one hyperoxaluric subject, 0.22 per cent of a glycine 1-C^{14} tracer appeared in urinary oxalate, a value four times the mean control value. In a second patient, the miscible pool of oxalate declined from 39.1 to 1.8 mg and its turnover from 109.7 to 8.2 mg per day during the study period. The nature of the metabolic defect in this patient, who incorporated glycine into oxalate normally, is obscure.

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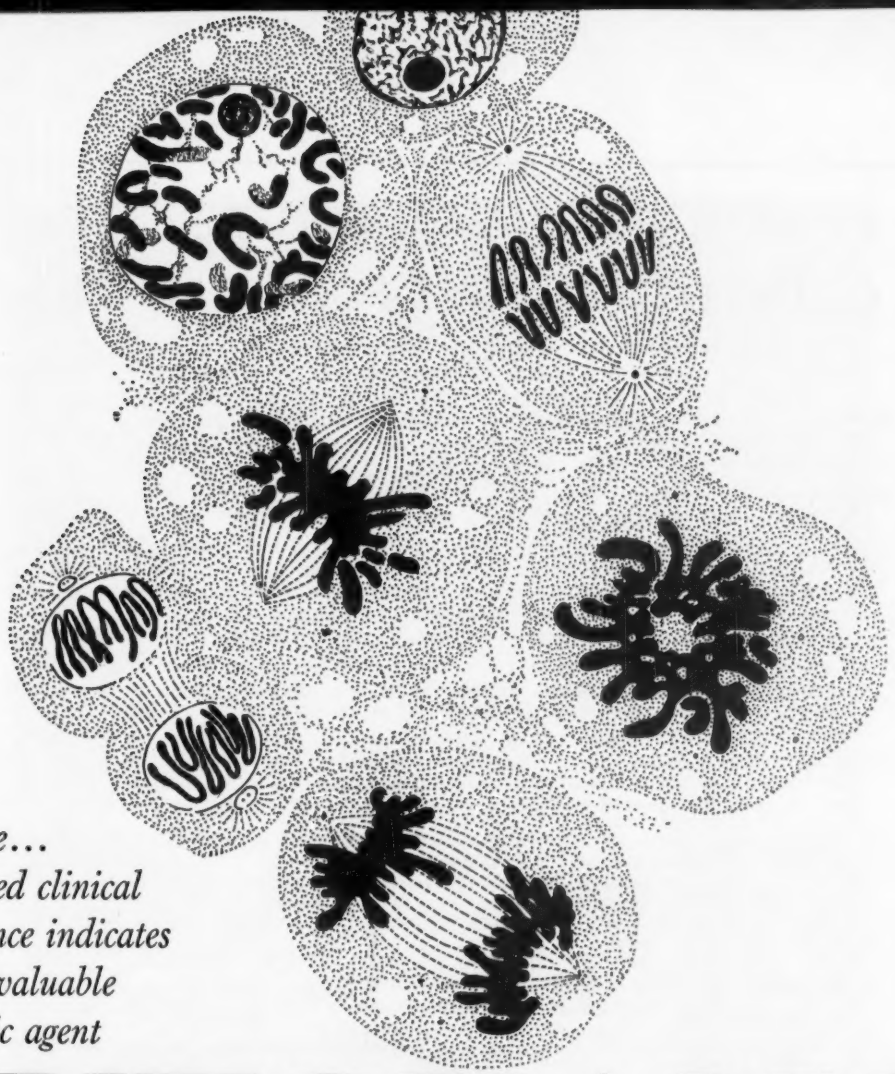

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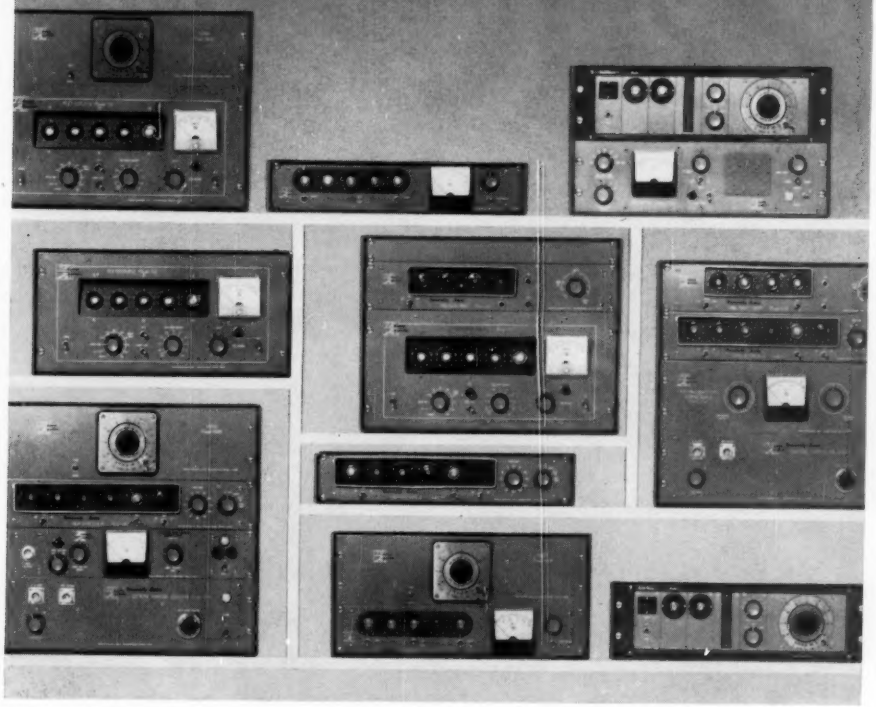

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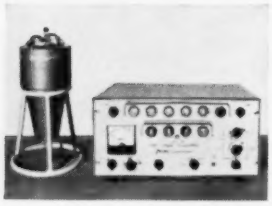

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